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THE UNIVERSITY OF ALBERTA

AN IMMUNOFLUORESCENT AND ELECTRON MICROSCOPIC EXAMINATION OF MEASLES VIRUS-INFECTED CELLS

by

PATRICIA A. TRYTTEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

VIROLOGY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

SPRING, 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled AN IMMUNOFLUORESCENT AND ELECTRON MICROSCOPIC EXAMINATION OF MEASLES VIRUS-INFECTED CELLS submitted by PATRICIA A. TRYTTEN in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in VIROLOGY.

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Date April 22, 1986

To my husband Tim.

Abstract

Measles virus (Halle strain)-infected Vero cells were studied by immunofluorescence and electron microscopy. Growth of the Halle strain at 39°C induced the temperature-dependent production of defective interfering particles which led to the establishment of persistently infected cells. In contrast, incubation of the infected cells at 32°C resulted in a lytic infection. A time sequence comparison was made of the lytically and persistently infected cultures.

Monoclonal antibodies directed against measles virus were used to detect viral specific proteins in infected cells. Nucleoprotein protein was detected as early as 6 hours post infection in lytically infected cells followed by the detection of polymerase (10 hours post infection), matrix (18 hours post infection), hemagglutinin (20 hours post infection) and fusion (21 hours post infection) proteins. The immunofluorescent staining patterns of the nucleoprotein and polymerase proteins were indistinguishable from each other as were the patterns of the hemagglutinin and fusion proteins. Matrix protein staining was unique from the other four proteins. Undiluted passage showed a noticeable decrease in the amount of immunofluorescent staining of the matrix, hemagglutinin and fusion proteins at 39°C as compared to 32°C. Discrete intranuclear fluorescent dots containing nucleoprotein and polymerase proteins were detected and the matrix-specific monoclonal antibody stained intranuclear material in a diffuse pattern. Budding virus was not seen in the undiluted passage at 39°C but was observed in the undiluted passage at 32°C. Typical viral nucleocapsid material was not observed in the nuclei of infected cells but simple and complex nuclear bodies were seen; perhaps depicting sites of viral protein accumulation and assembly of nucleocapsids.

The second undiluted passage of the Halle stain produced limited cytopathic effect at 39°C and a lytic infection at 32°C. The infection at 39°C displayed a cycling pattern of cytopathic effect. The cytopathic effect was characterized by the formation of syncytia, necrosis of the syncytia leading to holes or plaques in the monolayer and the subsequent "healing" of the plaque by the inward growth of the surrounding cells. These persistently infected cells contained measles virus antigens in multi-nucleated cells only. Decreased

amounts of five viral proteins were observed at 39°C compared to normal amounts in the 32°C cultures. The cells which filled in the syncytial plaques did not stain for the nucleoprotein, polymerase, matrix, hemagglutinin or fusion proteins.

This study reports for the first time the immunofluorescent detection of the polymerase and matrix proteins in the nuclei of measles virus-infected cells. The healing syncytium phenomenon observed in these persistently infected cells has previously been described only in nonproductive co-cultures derived from brain material of patients with subacute sclerosing panencephalitis. Transcriptional and/or translational blocks may be responsible for the decreased production of viral proteins in the undiluted passages of the Halle strain at 39°C compared to the lytic infection at 32°C.

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Abbreviations

C	Celsius
cAMP	3', 5' cyclic adenosine monophosphate
CDV	canine distemper virus
CF	complement fixation
cm	centimeter
CNS	central nervous system
CO_2	carbon dioxide
CPE	cytopathic effect
DI	defective interfering
DP	diluted passage
d pi	days post infection
d pp	days post passage
F	fusion / hemolysin
F_1	hemolysin subunit 1
F_2	hemolysin subunit 2
GAM-FITC	goat-anti-mouse antibody-fluorescein isothiocyanate
GAR-FITC	goat-anti-rabbit antibody-fluorescein isothiocyanate
H	hemagglutinin
h	hours
HA	hemagglutination
HAD	hemadsorption
HI	hemagglutination inhibition
HLI	hemolysis inhibition
HN	hemagglutinin - neuraminidase
h pi	hours post infection

IF	immunofluorescent
IM	infectious material
L	large protein of measles virus
LCMV	lymphocytic choriomeningitis virus
M	matrix protein (according to text)
M	molarity (according to text)
MEM	minimal essential media
mL	milliliter
mm	millimeter
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MV	measles virus
MV-H	measles virus-Halle strain
MV-L	measles virus-Lec strain
MW	molecular weight
NB	nuclear body
NC	nucleocapsid
NDV	Newcastle disease virus
nm	nanometer
NP	nucleoprotein
NT	neutralization
P	polymerase protein
PBS	phosphate buffered saline
pfu	plaque forming unit
RNA	ribonucleic acid
RP	ring plaque
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SSPE	subacute sclerosing panencephalitis
ts	temperature-sensitive
UP-1	undiluted passage - first
UP-2	undiluted passage - second
VSV	vesicular stomatitis virus
v/v	volume per volume
WEE	Western equine encephalitis virus
w/v	weight per volume

1. Introduction

1.1 The Disease Measles

Measles is a common childhood disease caused by the measles virus, a member of the genus Morbillivirus, family Paramyxoviridae. The disease is characterized by a maculopapular rash which persists for 3 or more days; a high fever; and a cough which may be accompanied by coryza and/or conjunctivitis (Centers for Disease Control. Morbidity and Mortality Weekly Report, 1983). Koplik spots may be found within the oral cavity. The resulting immunity is lifelong (Wild, 1981).

Measles virus was first isolated in 1954 by Enders and Peebles. This was followed by the development of an effective vaccine against measles (Enders et al., 1960) and its subsequent introduction into the health care system of developed countries beginning in 1960. Since that time, the number of clinical cases of measles has decreased dramatically in those countries with a measles vaccination program. Measles infection is still a major problem in areas without adequate vaccination programs and proper medical care. The number of global fatalities due to measles and its associated complications has been estimated to be as high as 1.5 million deaths per year (Marusyk, 1984).

1.2 The Measles Virion

The genus Morbillivirus consists of the measles virus (MV), canine distemper virus (CDV), rinderpest virus of cattle and pest-des-petits ruminants virus. The diseases produced by the morbilliviruses are characterized by a respiratory tract infection and cutaneous eruption. Canine distemper virus can produce a chronic neurologic disease in the dog and MV can produce a chronic neurologic disease in man. CDV, MV, and rinderpest virus are antigenically related, sharing nucleocapsid and envelope antigens (Örvell and Norrby, 1974). The morbilliviruses differ from other paramyxoviruses, ie., Sendai virus, simian virus 5, Newcastle disease virus, since morbilliviruses do not appear to attach to cell surface receptors containing neuraminic acid nor do they have any detectable virion-associated neuraminidase

activity.

The measles virion is an enveloped RNA virus containing a single-stranded, negative sense RNA genome of molecular weight 4.5 - 5.2 X 10^6 (Tucker et al., 1984; Baczko et al., 1984). The virus particle is spherical with a diameter of 100 to 300 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the measles virion has shown that it contains seven major proteins: large protein (L, 180-200,000 MW); hemagglutinin protein (H, 79,000 MW); polymerase protein (P, 72,000 MW); nucleoprotein (NP, 60,000 MW); hemolysin protein (F, 60,000 MW; which is cleaved to yield F_1 (41,000 MW) and F_2 (12,000-20,000 MW)); matrix protein (M, 37,000 MW); and cellular actin (A, 43,000 MW; Wechsler and Fields, 1978; Tyrrell and Norrby, 1978; Stallcup, Wechsler and Fields, 1979). All of these proteins, with the exception of actin, are virally-encoded. The actin is host cell-derived and incorporated into the virus particle during morphogenesis. The gene order from the virion genome 3' end is NP, P, M, F, H, and L (Richardson et al., 1985).

The H and F proteins are located on the viral surface and the other virus proteins (L, P, NP, M, A) are internal to the viral envelope (Fig. 1) as shown by trypsin treatment of the whole virions (Tyrrell and Norrby, 1978; Stallcup et al., 1979). The nucleocapsid (NC) constitutes the internal part of the virus and is composed of the genomic RNA (5%) surrounded by viral proteins (95%; Waters, Hersh and Bussell, 1972). Purified NC from virions contain the L, P, NP and M proteins and purified membranes from acutely infected cells contain the H, F, and M proteins (Stallcup et al., 1979).

The NP protein is phosphorylated and associated with the NC. It is the major viral protein in both the virion and infected cells and is the major structural component of purified NC (Hall and Martin, 1973; Mountcastle and Choppin, 1977). The NP protein of the morbilliviruses is thought to be the major cross-reacting antigen (Örvell and Norrby, 1974) although antigenic differences between the NP protein of MV and CDV have been shown with monospecific sera (Örvell and Norrby, 1980) and with monoclonal antibodies (Birrer, Bloom and Udem, 1981). The protein does appear to be antigenically stable within different strains of MV as no variation was detected between nine virus strains using a panel of six anti-NP

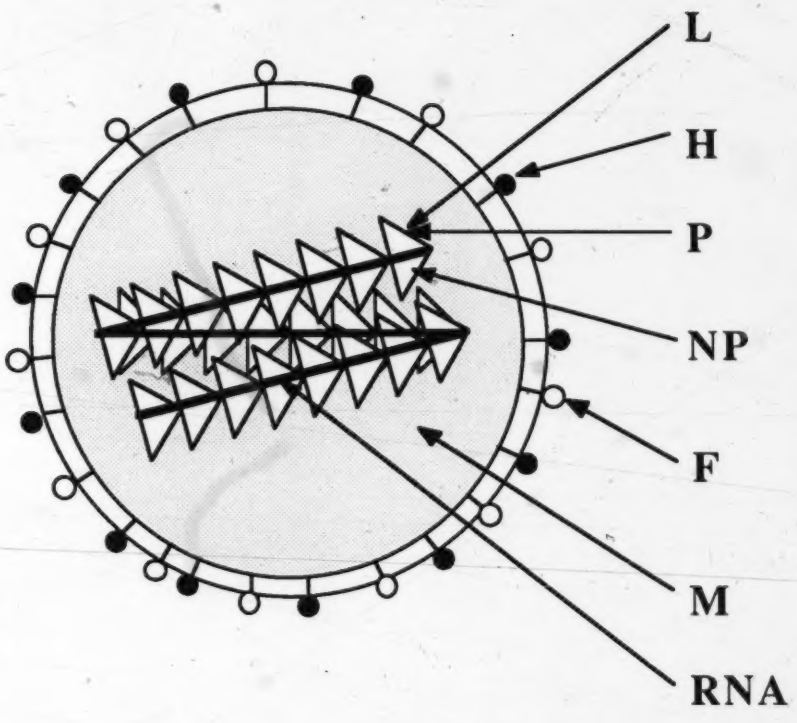


Figure 1.

Schematic representation of a measles virion showing probable location and association of structural components. L = large protein; H = hemagglutinin glycoprotein; P = polymerase protein; NP = nucleoprotein; F = fusion/hemolysin glycoprotein; M = matrix/membrane protein; RNA = virion genome.

monoclonal antibodies (Sheshberadaran, Chen and Norrby, 1983). The NP protein is found in the cytoplasm and can be found in the nucleus of persistently-infected cells (Wechsler and Fields, 1978; Norrby et al., 1982; Giraudon, Gerald and Wild, 1984).

The P protein is a phosphoprotein associated with the NC (Tyrrell and Norrby, 1978; Stallcup et al., 1979). The function of the P protein of morbilliviruses has not been defined but it is thought to be part of the transcription complex by analogy with other paramyxoviruses. In addition, the P protein may play a structural role in the NC (Wechsler and Meissner, 1982). The P protein is detected in the cytoplasm of infected cells by immunofluorescent procedures but has also been found in the nuclei by immunoprecipitation methods (Wechsler and Fields, 1978). A second overlapping reading frame within the P gene codes for a non-structural protein C, which is expressed in virus-infected cells (Bellini et al., 1985).

The immunological cross-reactivity of the P proteins of MV and CDV indicate their similarity (Örvell and Norrby, 1980). Within nine different strains of MV, the P protein appears to be antigenically stable when tested with a panel of six anti-P monoclonal antibodies (Sheshberadaran et al., 1983).

The large L protein is a minor component of the measles virus (Mountcastle and Choppin, 1977). Some researchers have found the L protein to be associated with purified NC (Stallcup et al., 1979) while others have not detected this association (Tyrrell et al., 1980). Along with the P protein, the L protein is thought to be part of the transcription complex, and has been identified in the cytoplasm and nuclei of MV infected cells (Wechsler and Fields, 1978).

The MV envelope consists of a lipid bilayer which is obtained from the host-cell plasma membrane during viral budding. The cellular membrane proteins are largely replaced by three viral-coded proteins: the H and F glycoproteins, and the nonglycosylated M protein (Dubois-Dalcq, Holmes and Rentier, 1984). The glycoproteins are trans-membrane proteins which form spike-like projections approximately 10 nm long on the virion surface. The F glycoprotein is anchored in the lipid bilayer by a hydrophobic region at the C-terminal end of

the protein (Dubois-Dalcq, Holmes and Rentier, 1984) and the H glycoprotein is anchored by a hydophobic region near the amino terminus (Alkhatib and Briedis, 1986). The M protein does not span the membrane but is associated with the inner surface of the lipid bilayer.

The H (hemagglutinin) protein is the major glycoprotein of MV (Mountcastle and Choppin, 1977; Tyrrell and Norrby, 1978; Vainionpää, Ziola and Salmi, 1978). The functional H glycoprotein of MV is present as a dimer (150,000 - 160,000 MW) and detected by SDS-PAGE under non-reducing conditions (Rima, 1983). It possesses the receptor-binding activity which is responsible for the attachment of the MV to host cell receptors and red blood cells. Paramyxoviruses other than the morbilliviruses also carry a neuraminidase activity associated with this gylcoprotein (HN), which can destroy the neuraminic acid-containing receptors for the virus. Environmental conditions such as chloride ion concentration and pH, may regulate the two opposing activities of this HN glycoprotein, namely receptor binding and destruction (Choppin et al., 1981). At this time, it is controversial whether or not the receptors for morbilliviruses contain neuraminic acid. Although the HN gylcoprotein mediates the important first step in infection, namely virus adsorption, it does not determine host range (Choppin et al., 1981). The rationale being that most vertebrate cell surfaces contain macromolecules containing neuraminic acid, but not all these cells can be infected by paramyxoviruses. According to Choppin et al. (1981) and Dubois-Dalcq et al. (1984) the susceptibility of the F_0 protein to cleavage by a host cell protease is a major determinant of virus host range and virulence.

Monospecific polyclonal sera prepared against the H protein of MV contains neutralizing (NT) and hemagglutination inhibition (HI) activity (Örvell and Norrby, 1980). Monoclonal antibodies directed against the H protein also show NT and HI activity and rarely hemolysis inhibition (HLI) activity (Togashi et al., 1981; Norrby et al., 1982). Three epitope regions have been assigned to the H protein on the basis of competitive binding assays with monoclonal antibodies (Carter et al., 1982; Sato, Fukuda and Sugiura, 1985). Sheshberadaran et al. (1983) reported that eleven anti-H monoclonal antibodies could be divided into at least nine differently reacting binding groups. The use of monoclonal antibodies has also led to the

detection of antigenic variation in the H proteins of various strains of MV (ter Meulen et al., 1981; Sheshberadaran et al., 1983). Cross-reaction of the H proteins of MV and CDV occurs only to a limited degree (Örvell and Norrby, 1980).

The other surface glycoprotein of MV is the fusion (F) protein. It is responsible for virus entry into the host cell via fusion of viral and cell membranes and also functions to fuse infected cell membranes thereby producing multi-nucleated cells (syncytia). Post-translational cleavage of the F_0 glycoprotein to its biologically active form (F), yields the F_1 and F_2 subunits which are held together by disulfide bonds. Cleavage by a host cell protease occurs mainly when the glycoprotein is at or near the plasma membrane (Dubois-Dalcq et al., 1984). For example, the F_0 glycoprotein of NDV is cleaved in the lumen of the rough endoplasmic reticulum, while Sendai virus F_0 protein is cleaved at the plasma membrane (Dubois-Dalcq et al., 1984). The F_2 subunit is the portion which is glycosylated (Mountcastle and Choppin, 1977).

Antiserum prepared against the purified 41,000 MW MV protein precipitates the F_1 proteins of MV and CDV but not the F_2 proteins (Örvell and Norrby, 1980). Monoclonal antibodies specific for the F component can immunoprecipitate both the F_1 and F_2 proteins and contain HLI activity (Norrby et al., 1982) supporting the role of F protein in hemolysis. Competitive binding assays with anti-F monoclonal antibodies indicate four antigenic sites (Sato et al., 1985) and six antigenic sites (Shesheradaran et al., 1983) for the F protein.

Antibodies to the F protein halt the spread of paramyxovirus infection via membrane fusion whereas anti-H antibodies do not prevent this spread but do neutralize released virus. The importance of an immunological response to the F protein was seen in individuals which received a formalin-killed MV vaccine and later developed atypical measles. The formalin treatment destroyed the antigenicity of the F protein but not the H protein (Norrby, Enders-Ruckle and ter Meulen, 1975). The recipients of this vaccine had an antibody response to all the MV proteins except the F protein. It has been proposed that when these individuals were subsequently exposed to MV, some respiratory tract cells were infected and the infection spread to adjacent cells by cellular fusion due to a lack of anti-F antibodies (Choppin et al.,

1981). The patients contracted an atypical MV infection even in the face of a secondary immune response to all the MV proteins except the fusion protein.

The M (matrix/membrane) protein is a non-glycosylated protein which is associated with the inner surface of the viral envelope and with the NC. The M protein is believed to play a key role in the assembly of budding viruses where it is thought to align the NC beneath the areas of the cell membrane that contain the H and F glycoproteins. Interruptions of this process are believed to be important in the mechanism of MV persistence.

The affinity of the M protein for the NC and glycoproteins of paramyxoviruses has been well documented. Stallcup et al. (1979) found the M protein of MV to be present in both membrane and NC preparations, the latter constantly contaminated with M protein. Studies with an anti-M monoclonal antibody also indicate that the M protein is associated with the NC in MV infected cells (Norrby et al., 1982). Electron microscopic examination of Sendai virus NC, isolated by a chloroform-methanol extraction procedure, revealed a sheath-like cover composed of small globular particles of M protein (Yoshida et al., 1976). Selective aggregation of viral components in vitro demonstrated that the NC protein does not combine with viral glycoproteins unless M protein was present (Yoshida et al., 1976).

Rabbit antisera prepared against MV and CDV cross-react with the M proteins of both viruses (Hall et al., 1980). Örvell (1980) prepared a monospecific serum against the M protein of CDV and found that it precipitated the M protein of MV. Recent evidence has shown that the epitopes on the M protein of different strains of MV showed extensive variation in their reactivity with a panel of anti-M monoclonal antibodies (Sheshberadaran et al., 1983). Nine MV strains were tested with a panel of nine anti-M monoclonal antibodies using the techniques of radioimmune precipitation assay (RIPA) and enzyme-linked immunosorbent assay (ELISA). The antibody panel represented at least six independently reactive groups (epitopes). It was postulated that a larger bank of anti-M monoclonal antibodies could be used to differentiate between MV strains based on the pattern of occurrence of M epitopes (Sheshberadaran et al., 1983).

Another major protein of the measles virion is cellular actin. Actin may play a role in the viral budding process since chemical inhibitors which interfere with actin filaments seem to decrease the number of budding MV but not the amount of intracellular MV production. MV specific antibody added to the surface of live MV infected cells causes the redistribution of cell surface viral glycoproteins into a patch or "cap". The association of M protein with the transmembrane viral glycoproteins explains why the M protein is also observed to cap, a process called "co-capping". When cytochalasin B, an inhibitor of actin polymerization, is added to infected cells, only the co-capping of the viral envelope glycoproteins and M protein is observed in contrast to the normal co-capping of envelope glycoproteins, M protein and nucleocapsid structures (Tyrrell and Ehrnst, 1979). It appears that the cytochalasin B interfers with the linkage, possibly by actin filaments, which directs and aligns the nucleocapsids with the membrane budding sites (Tyrrell and Ehrnst, 1979). Stallcup et al. (1983) hypothesized that contraction of the actin micro filaments may cause cleavage of the viral bud from the cell membrane.

1.3 The Disease SSPE

A rare complication of measle virus infection is the disease subacute sclerosing panencephalitis (SSPE, Dawson's encephalitis, van Bogaert's leukoencephalitis). About 1 case of SSPE occurs per million cases of measles. SSPE is a slowly progressing disease of the central nervous system (CNS) of children and young adults. This disease is one of the best studied examples of a persistent infection in man caused by a virus which normally produces an acute, self-limiting disease. Inflammatory lesions in both the white and grey matter of the brain (Johnson, 1970) and typical eosinophilic intranuclear inclusions (Dawson, 1933) characterize this fatal disease. Disease symptoms beginning with a decrease in intellectual skills, progress to extensive loss of cortical function and death. Epidemiological data from numerous countries has shown that SSPE patients often contracted measles at a young age; the mean age being less than 2 years. In comparison, the mean age for American children contracting measles was 4 years (Wechsler and Meissner, 1982) and is currently closer to 10

years of age. The average interval of time between measles and the early symptoms of SSPE is 6-7 years (Wechsler and Meisner, 1982). It is believed that measles virus survives in a non-lytic persistent state in the brain cells after the initial attack of measles.

SSPE patients have unusually high measles antibody titers in their serum and cerebrospinal fluid (CSF) as measured by neutralization, hemagglutination-inhibition, and complement fixation (CF) tests (Connolly et al., 1967). This hyperimmune oligoclonal response to MV structural proteins, which is not seen in convalescent sera from measles cases, supports the idea that the immune system of SSPE patients has been exposed to MV antigens for years.

1.3.1 Cytopathology of SSPE

The first proposal that SSPE might be caused by a virus was based on the observation of eosinophilic intranuclear inclusion bodies in nerve cells of SSPE cases (Dawson, 1933; Cowdry, 1934). Nerve cells do not normally contain inclusion bodies and certain viruses can cause the formation of eosinophilic nuclear inclusions. Electron microscopic investigation later showed that these inclusions consisted of structures resembling paramyxovirus nucleocapsids (Bouteille et al., 1965; Tellez-Nagel and Harter, 1966). Measles antigens have been detected in SSPE brain material by immunofluorescent procedures (Connolly et al., 1967) and immunoelectron microscopy (Jenis et al., 1973).

Initial attempts to isolate the virus from brain tissue were unsuccessful, presumably because the virus was cell-associated and structurally incomplete. Co-cultivation of the brain cells with cells known to support measles virus replication (ie. HeLa, monkey kidney cells) resulted in viral isolations (Payne, Baublis and Itabashi, 1969; Horta-Barbosa et al., 1969) approximately 20% of the time (Wechsler and Meissner, 1982). As well, the co-cultivation of lymph nodes from an SSPE patient was found to yield measles virus (Halle strain; Horta-Barbosa et al., 1971). The rescued viruses may produce either a lytic infection (lytic SSPE viruses) of the permissive tissue culture cells or a persistent infection in which little or no infectious virus is produced and the virus remains cell-associated (cell-associated SSPE;

Doi et al., 1972; Dubois-Dalcq et al., 1974; Burnstein et al., 1974; Sheppard et al., 1975). A few of the cell-associated SSPE isolates produce an unusual cytopathic effect (CPE) in which the syncytia (multinucleated cells) do not progress but detach from the glass leaving a hole or plaque in the monolayer (Mirchamsy et al., 1978). In some persistently-infected cells, this hole has been observed to "heal" by the inward growth of the neighbouring cells (Burnstein et al., 1974; Makino et al., 1977; Norrby et al., 1985).

1.3.2 SSPE - The M Protein Controversy

Although the molecular basis of MV persistency in SSPE brain tissue is not known, accumulated evidence suggests that viral assembly is defective in these CNS cells. Viral NC accumulates in the cytoplasm and nucleus of infected brain cells but does not align under the plasma membrane and no viral budding is observed. Generally, MV M protein is not detected in SSPE brain specimens (Hall and Choppin, 1981; Haase et al., 1985; Shapshak et al., 1985) though exceptions have recently been reported (Norrby et al., 1985).

A combination of immune precipitation and Western blotting was used to show that the brain tissue of SSPE patients contained all MV proteins except the M protein (Hall and Choppin, 1981). This result supported the earlier finding that all MV proteins, except the M protein, could be detected in an explant culture of SSPE brain cells (Hall and Choppin, 1979). Johnson et al. (1981) found that the M protein, but not the NP protein, disappeared from the brains of their hamster models of SSPE. The disappearance of M protein coincided approximately with the in vivo change from a productive infection to a cell-associated viral form and also with appearance of serum antibody to MV. Since the M protein plays a key role in virus assembly, this apparent lack of M protein would explain why budding virus is not observed in SSPE brain tissue and why infectious virus cannot be directly isolated by conventional methods.

More recently, Haase et al. (1985) detected NP antigen, but not M antigen, in SSPE brain material using monoclonal antibodies and an avidin-biotin-peroxidase labelling technique. The brain cells had depressed levels of plus- and minus-strand viral RNA as shown

by in situ hybridization using NP and M gene probes (Haase et al., 1985). Shapshak et al. (1985) also found no evidence of M protein in two SSPE brains by immune precipitation and immune blotting but did detect both the M and NP gene nucleotide sequences by in situ hybridization techniques on cryostat sections of SSPE brain. The absence of M protein was therefore not due to the deletion of the M gene (Shapshak et al., 1985).

Measles virus M protein has been detected in brain sections of four patients with SSPE by immune fluorescence with monoclonal antibodies (Norrby et al., 1985). Nine monoclonal antibodies representing six different epitopes on the M protein were used. All four cases stained for all five antigens (NP, P, H, F and M). In three cases, eight or nine anti-M antibodies gave a moderate-strong staining. In the other case, five antibodies gave only a weak staining, two antibodies gave moderate staining and no visible staining was seen with two anti-M antibodies, indicating antigenic diversity of the M protein detected in SSPE brain. The M protein detected may have been incomplete or nonfunctional (Norrby et al., 1985).

Initially, it was reported that SSPE patients lacked serum antibodies specific for the M protein of MV but had antibodies directed against other MV proteins (Hall, Lamb and Choppin, 1979; Wechsler, Weiner and Fields, 1979) as determined by immunoprecipitation experiments. Recently, antibody to the M protein has been detected in SSPE sera (Ohara et al., 1985) and CSF specimens (Karcher et al., 1982). Ohara et al. (1985) compared ten different solubilizing buffers that were reported in the literature and found immunoprecipitation results to vary widely. The detection of M protein was largely influenced by the kind of buffer used, which may explain the disagreement concerning the presence of anti-M antibody in the sera of SSPE patients (Ohara et al., 1985).

In agreement with the SSPE brain results, M protein has been found to be lacking in some cell-associated SSPE cell lines (SSPE brain co-cultivations), and present in others. Several cell-associated SSPE cell lines do not contain detectable M protein (Lin and Thormar, 1980; Stephenson, Siddel and ter Meulen, 1981; Carter, Willcocks and ter Meulen, 1983; Sato, Fukuda and Sugiura, 1985). Stephenson et al. (1981) observed the synthesis of H, NP and possibly F protein in lytic and persistent infections with the Lec strain (SSPE isolate) but M

protein synthesis was only detected in the lytic infection. In vitro translation of mRNA isolated from both the lytic and persistent infection directed the synthesis of H, NP and M proteins, suggesting a translation block for the M protein in the persistent infection (Stephenson et al., 1981). In agreement with these findings were the results of Carter et al. (1983) who determined that the mRNA for the M protein was present in the cell-associated SSPE cell line (N-1; SSPE brain co-cultivated with Vero cells) but could not detect M protein. It was not found by either immunofluorescence or RIA using four anti-M monoclonal antibodies or immunoprecipitation or in vitro mRNA translation. Carter et al. (1983) postulated that the defect could be due to premature termination, failure to initiate translation or a frameshift mutation.

In the cell-associated SSPE cell lines where M protein has been observed, it appears to be unstable and susceptible to rapid degradation within the cell (Sheppard et al., 1985). The matrix protein is thought to undergo rapid post-translational degradation in the IP-3-Ca cell line (SSPE brain co-cultivated with BSC-1 cells) and is only detected with short labelling periods (Sheppard et al., 1985). The M protein was also found to be unstable in a MV persistently-infected HeLa cell line (Young, Heineke and Wechsler, 1985). Roux and Waldvogel (1982) studying Sendai virus persistently-infected hamster kidney cells, demonstrated by pulse-chase experiments that the M protein was synthesized at a normal rate, but was unstable compared to the other viral proteins.

In summary, it appears that the mRNA message for the M protein is present in SSPE brain cells, cell-associated SSPE cells and some persistently infected cells. If the M protein is translated, it appears to be unstable and may be nonfunctional.

1.4 Viral Persistence

MV persistence of tissue culture cells is of current interest since MV is known to chronically infect the nervous system of SSPE patients and has been implicated as a possible causative agent in multiple sclerosis (MS). Besides MV, other enveloped RNA viruses can cause persistent infection in tissue culture cells (Rima and Martin, 1976). These include such

viruses as HVJ (Sendai) virus, Newcastle Disease Virus (NDV), parainfluenza virus type 3, mumps virus, vesicular stomatitis virus (VSV), rabies virus, Japanese encephalitis virus, Western equine encephalitis virus (WEE), influenza virus A, Sindbis virus, and arena viruses such as Parana virus and lymphocytic choriomeningitis virus (LCMV).

1.4.1 Common Properties Of Persistent Infections

Persistent infections of tissue culture cells are characterized by a number of common properties (Rima and Martin, 1976). Most persistent infections release infectious virus but some are non-productive as determined by plaque assay. Levels of released infectious virus may fluctuate during the subculturing of the cells, often coinciding with variations in the appearance of CPE and cell destruction. The cyclic expression of antigen and infectious virus was documented in a baby hamster kidney cell line persistently-infected with an SSPE strain (Lec) of MV (Cremer, Oshiro and Hagens, 1979).

A second property of persistently-infected cells, as demonstrated by immunofluorescence, is that usually a large percentage of the cells contain viral antigens. In some virus/cell systems, the percentage of fluorescing cells undergoes cyclic variations (Ehrnst, Weiner and Norrby, 1974; Cremer et al., 1979; May and Menna, 1979). Ehrnst et al. (1974) demonstrated a cyclic variation of the amount of membrane fluorescence in measles/Lu 106 cells which displayed less fluctuation in intracellular antigen and hemadsorption. Persistently-infected cells may carry viral antigen on the cell surface as well as in the cytoplasm and nucleus.

Another property of persistently infected cells is immunity to super-infection by homologous virus but not to heterologous viruses. This means that the cells will support the growth of unrelated viruses but when superinfected with a related virus, no CPE or transient CPE is observed and no infectious virus is released. Rustigian (1966) found that measles/HeLa cells were resistent to superinfection by MV, but were able to support the normal growth of vaccinia virus, herpes virus and polio virus. Superinfecting virus is able to attach to persistently-infected cells (Rustigian, 1966) and occasionally the development of

CPE, which does not progress, is seen. The observations imply that the superinfecting virus can enter the cells and start the infection process but viral maturation and spread is blocked (Rima and Martin, 1976).

Persistently-infected cells cannot be "cured" of viral antigens by subculturing in the presence of specific antiserum, although this procedure may select for non-yielding cell lines. Rustigian (1962) discovered that incubation of MV persistently-infected cells in the presence of measles antibodies often produced persistently-infected cells which no longer released infectious virus (non-yielder state) but still contained viral antigen by immunofluorescense. Whether these non-yielders are truly not releasing infectious viruses or perhaps are releasing a labile virus, temperature-sensitive (ts) mutant or defective-interfering virus which is not detected, remains to be determined (Rima and Martin, 1976).

It appears that viral replication in persistently-infected cells is generally blocked at one of the last stages of maturation. This is supported by the observations of MV persistently-infected cells which contain large amounts of viral antigen and nucleocapsid-like structures (Dubois-Dalcq et al., 1974). Inhibitors of protein and RNA synthesis did not inhibit the release of extracellular MV from persistently-infected HEF cells when they were co-cultivated with BSC-1 cells indicating that the necessary viral proteins and RNA were present in the persistent HEF culture (Knight et al., 1974). Properties of both host cell and virus appear to be important in the establishment and maintenance of persistence.

1.4.2 Physiological Factors Influencing Persistent Infections

Certain paramyxoviruses interact very differently with various cell lines. For example, infection by the Halle strain of MV led to a lytic infection of Vero cells but gave rise to a persistent infection of BGM cells; both are African green monkey kidney cell lines (Wild and Dugre, 1978). Wild and Dugre concluded that a host-cell factor must play a role in the restriction of virus replication. This conclusion was supported by Cremer et al. (1979) who developed a BHK cell line (0-853) persistently-infected with the Lec strain of MV. Infection of other cell lines (Vero, CV-1, HFDL and BHK_{21}) resulted in complete destruction of the

monolayers within one to three passages.

There has been some evidence to suggest that even the stage of cell growth is important in the establishment and maintenance of some persistent infections. The Schwarz vaccine strain of MV was found to produce a latent infection in nonproliferating hamster embryo fibroblast cells and a lytic infection in actively proliferating hamster cells (Haspel et al., 1973). The opposite was seen in a mumps virus/conjunctiva cell persistent infection; virus was released when the cells entered a nonproliferating state (Rima and Martin, 1976). Ehrnst et al. (1974) proposed that factors such as cell growth, phases of the cell cycle, and energy generating systems may play a role in the maintenance of persistent infection. Numerous studies have shown that addition of 3', 5' cyclic adenosine monophosphate (cAMP) to tissue culture media, markedly affects the replication of MV in human and primate cells (Robbins and Eagle, 1985). These effects include decreased production of infectious particles, inhibition of virus-mediated cell fusion, altered morphology and distribution of cytoplasmic nucleocapsids, impaired virion assembly, and inhibition of viral protein and RNA synthesis. Robbins and Eagle (1985) found that MV persistently-infected human cells (AV3A1/MV) exposed to 1 millimolar cAMP for 4 days showed a dramatic increase in the percentage of cells having intranuclear virus inclusions and a twofold increase in the number of inclusions per invaded nucleus. Since the effects observed in cAMP-treated MV-infected cells are similar to processes seen in SSPE brain and SSPE persistently infected cell lines, and the fact that CNS tissue has relatively high intracellular cAMP levels, it has been postulated that cAMP (or events mediated by cAMP) may be involved in modulating MV replication in SSPE (Robbins and Eagle, 1985). In contrast, interferon probably does not play a controlling role in the maintenance of persistent infections as indicated by the ability of persistently-infected cells to support the growth of heterologous viruses. Interferon is not detected or found in low levels in a variety of persistently-infected cell lines.

The virus-specific factors which are believed to be important in the establishment and maintenance of persistence are temperature-sensitive (ts) mutants and defective-interfering (DI) particles. These may act alone or in concert with one another to lower the virulence of

the carried virus and promote cell survival.

1.4.3 Defective Interfering Particles

Huang and Baltimore (1970) coined the term defective interfering (DI) particles to describe the biologically active defective viral particles produced by many animal viruses. The DI particles have normal viral structural proteins but contain only a part of the viral genome. These particles are "defective" in that they cannot replicate if the wild-type or standard virus is absent. DI particles interfere specifically with the intracellular replication of the non-defective homologous virus (standard virus). The molecular mechanism of autointerference is not known but is believed to be related to competition between the DI genomes and standard genomes for the replicative enzymes and NC structural proteins which are supplied by the standard virus. A small DI genome will be replicated more quickly than the nondefective virus genome if the rates of RNA synthesis on both templates are the same (Hsu et al., 1985). The greater number of DI genomes will compete with the standard genomes for NC proteins and enzymes. It is clear that the DI particle genomes are efficiently replicated by the enzymes provides by the standard virus and the standard virus also provided other missing gene products (Kang, Schubert and Lazzarini, 1985). Therefore DI particles effectively interfere with the replication but not the transcription of standard Sendai virus (Carlsen, Peluso and Moyer, 1985). Also the DI particles are able to increase in number relative to the yield of standard virus, a process called "enrichment" (Huang, 1973). Enrichment and interference may result from preferential replication of the DI genomes due to an increased number of initiation sites or higher affinity initiation sites on the DI genomes. The best studied DI particles are those of VSV which differ in length from the standard virions and are readily separated by rate zonal centrifugation. Many animal viruses produce DI particles. These include such viruses as rabies virus, Sendai virus, CDV, MV, WEE, Japanese encephalitis virus, NDV, influenza virus, Rift Valley fever virus, simian virus 40, LCMV, polyoma virus and polio virus.

Von Magnus (1954) first described "incomplete" particles produced by inoculating fertilized eggs with high multiplicities of influenza virus. The multiplicity of infection (moi) used in passaging virus affects the outcome of infection. Passaging MV in an undiluted form (UP virus) resulted in "strand" type CPE and the appearance of auto-interfering activity, whereas the inoculation of diluted virus material (DP virus) produced spreading syncytia formation and no auto-interference (Norrby, Chiarini and Marusyk, 1970). Short nucleocapsids with small RNA strands were found in cells infected with UP measles virus (Kiley, Gray and Payne, 1974). Kiley et al. (1974) suggested that under certain conditions MV may produce "defective" or "sub-genomic" virus components. Undiluted passage of MV produced both a rapidly sedimenting infectious virus containing 52S RNA and a DI particle which sedimented more slowly and contained 18S RNA (Hall, Martin and Gould, 1974). Rima, Davidson and Martin (1977) found that they could establish persistent infection of Vero cells using undiluted Edmonston virus stocks which had low infectivity, the ability to cause interference, and normal hemagglutinin titers. They determined that their persistent infections were likely due to the presence of DI particles rather than virus mutants. It is now generally acknowledged that several undiluted passages of a virus stock will produce DI particles.

The majority of VSV DI particles are composed of 5' half of the genome but DI particles composed of the 3' end of the genome have also been found (Kang et al., 1985). The terminal promoter sequences essential for RNA replication are always conserved in DI particles (Kang et al., 1985). A well-characterized VSV DI particle which contained the 3' half of the parental genome, was found to be lacking most of the polymerase L gene. Since the 3' end was conserved, it could be transcribed into four functional mRNAs; this synthetic activity being unique among DI particles (Kang et al., 1985).

In contrast, the majority of Sendai virus DI particles contain internally deleted RNA molecules called fusion DI RNA (Re, Morgan and Kingsbury, 1985). The internal genes are deleted due to errors in viral RNA replication and various amounts of 3' terminal end (NP gene) are fused with various amounts of the 5' terminal end (L gene). This type of deletion

mutant seems to be unique to Sendai virus among the nonsegmented, negative sense RNA viruses (Re et al., 1985). These authors suggested that these DI genomes were generated by a copy-choice mechanism, in which the polymerase jumped during the replication of the virus genomes. The regions that flank each of the deletion sites have been sequenced, showing similarities that mimic viral RNA polymerase control signals (Re et al., 1985). The other type of Sendai virus DI particle contains sequences representing only the 5' end of the L gene (Carlsen et al., 1985). Retaining only 11% of the standard virus genomic RNA, the Sendai virus DI-H particle represents a portion of the L cistron and contains copyback termini with a 3' terminus complementary to the 5' terminus (Carlsen et al., 1985). This DI-H particle is thus defective in transcription and needs the standard Sendai virus as helper to provide the necessary mRNAs and proteins.

Cyclic changes in the amount of DI and infectious virus have been documented in serial undiluted passages of VSV (Huang, 1973; Palma and Huang, 1974). A graphic illustration of this cyclic phenomenon is the formation of "ring plaques" under plaque assay conditions (Pfau, Welsh and Trowbridge, 1973; MacDonald, 1978; Tsang, Chang and Marusyk, 1981). Neutral red staining of ring plaques shows alternating, concentric rings of living and dead cells. The areas of dead cells represent the part of the cycle with maximum complete infectious particle production and the areas of living cells represent the time of the cycle when infectivity is low and DI particle production is high. The Halle strain of MV (SSPE isolate) produced large ring plaques at 37°C containing multiple alternating rings of living and dead cells, but incubation at 39°C or 32°C did not produce ring plaques (Tsang et al., 1981). This phenomenon was associated with the temperature-dependent production of DI particles which were purified by linear gradient centrifugation and found to interfer with the replication of standard measles virus but not VSV (Tsang et al., 1981). Infectious pancreatic necrosis virus (IPNV) produced ring plaques characterized by a furry border containing living cells within the boundaries of the plaque (MacDonald, 1978). MacDonald (1978) concluded that the production of IPNV ring plaques correlated with the production of DI particles. LCMV was also found to produce ring plaques depending on the partial pressure of the

carbon dioxide (pCO_2) in the growth atmosphere (Pfau et al., 1973). This observation suggests that the metabolic state of the cell plays a role in the manifestation of the interference phenomenon (Pfau et al., 1973) in this virus-cell system.

In addition to the cyclic changes in the amount of DI and infectious VSV virus observed in tissue culture cells, a cycling pattern of VSV has also been observed in mouse brains (Cave, Hendrickson and Huang, 1985). Mice were inoculated intranasally with a mixture of 10^8 pfu of standard VSV and 10^5 pfu equivalents of DI particles (Cave et al., 1985). A cycling pattern of VSV standard virus and DI particles was observed along with the detection of DI RNA in the brains several days after inoculation. Cave et al. (1985) suggested that there was a dynamic continuous interaction between the DI and standard virus particles beyond the initial replication site as the virus population spread into the animal. Cyclic production of DI particles which contain functional genes may produce quantitative differences in the expression of certain genes (Cave et al., 1985).

In summary, DI particles modulate viral infections in vivo and in vitro by interfering with the replication of the nondefective standard virus. The production of DI particles can lead to the establishment of persistence in tissue culture cells and may be a controlling factor in human persistent infections such as SSPE.

1.4.4 Temperature-Sensitive Mutants

The involvement of temperature-sensitive (ts) mutants in persistent infection is based on two types of experiments (Rima and Martin, 1976). Firstly, persistently-infected cells at 37°C spontaneously release virus containing ts mutations. The second type of experiment involves incubation of persistent infections at lower temperatures leading to an increase in the amount of extracellular virus, which in some cases appears to be temperature-sensitive. Viruses recovered from persistent cultures often differ from the parental virus in such biological properties as virulence, plaque size, and antigenicity. Preble and Youngner (1975) suggested that selection of ts mutants may be involved in the establishment and/or maintenance of persistent infections both in cell culture and animals. Mouse L cells

persistently-infected with NDV (NDVo/L) have been extensively studied by Preble and Youngner (1975). A small plaque mutant called NDVpi was isolated from NDVo/L cells. The mutant was less virulent for mice and embryonated eggs than the wild-type virus (NDVo). NDVpi differed from NDVo in a number of other biological properties, including thermal stability of HA activity and neuraminidase enzyme and growth pattern in L cells. The plaque-type marker and the ts marker were found to be independent mutations since ts(+) revertants still retained the small plaque phenotype (Preble and Youngner, 1975; Haspel et al., 1973). Ten independently isolated clones of NDVpi were ts mutants with 41-42°C as a non-permissive temperature. At this high temperature, all clones showed defects in RNA polymerase activity and were therefore defective in RNA synthesis (Preble and Youngner, 1975). Restricted viral RNA synthesis was responsible for the rapid establishment of persistence in Vero cells by a ts mutant (ts-23) of Sendai virus (Kanda and Shibuta, 1982). The mutant has a temperature-sensitive defect in RNA polymerase activity; 31°C and 39°C are the permissive and non-permissive temperatures, respectively.

MV persistently-infected hamster embryo fibroblast cells were found to release one hundred times more virus at 33.5°C compared to incubation at 37°C and no detectable virus at 39°C (Haspel et al., 1973). The virus was temperature sensitive if grown at 39°C and generally less virulent than virus released by co-cultivation of the persistently-infected cells with BSC-1 cells. Gould and Linton (1975) studying a MV persistent infection of Hep_2 cells, noted that the virus released at 37°C was temperature sensitive at 39.5°C in contrast to the wild-type virus. Shift of the measles/Hep_2 cultures from 37°C to 39.5°C resulted in the disappearance of most surface antigens within 24 h whereas the intracellular virus antigens did not totally disappear until the fifth passage.

The selection of viral mutants and the cyclic production of DI particles in persistent infections are not mutually exclusive events (Preble and Youngner, 1975). In fact, DI particles appear to be functioning in the NDV/L cell and NDV/BHK cell persistent infections. Preble and Youngner (1975) hypothesized that even in a virus/cell system in which DI particles predominate initially, evolution of mutants, particularly ts mutants, may be

involved in the maintenance of the persistence.

1.5 Cytopathology of MV Infected Cells

1.5.1 Immunofluorescence Studies

Hybridoma technology (Köhler and Milstein, 1975) has enabled researchers to produce monospecific antibodies against the structural components of MV (Togashi, Örvell, Vartdal and Norrby, 1981; Giraudon and Wild, 1981). In combination with immunolabelling techniques such as immunofluorescence, the intracellular occurrence and distribution of the various MV components have been observed using monoclonal antibodies. This approach has been used to compare the amounts of selected MV proteins in productive and nonproductive infections.

Different immunofluorescent stained appearances have been observed for the various components of MV (Norrby et al., 1982). Antibodies against NP and P proteins stained cytoplasmic inclusions varying in size from small dots to confluent masses, frequently in a perinuclear position in lytically infected cells (0.01 pfu/cell) at 2 to 4 d pi (Norrby et al., 1982). Occasionally the nuclei of infected cells contained distinct dot-like inclusions which stained with the NP monoclonal antibody. This observation of intranuclear NP protein has been reported by others (Giraudon, Gerald and Wild, 1984) and is usually associated with persistent infections although it can be seen in lytic infections also. These same nuclear dots have also been stained with antibody against the C protein of MV in both persistently-infected (MA 160 SSPE) and lytically infected (Edmonston) cells (Bellini et al., 1985). The staining pattern obtained with the anti-L monoclonal antibody was similar to the NP and P staining pattern (Giraudon et al., 1984). Norrby et al. (1982) reported that the M monoclonal stained both cytoplasmic granular material, especially in the centre of syncytia, and cytoplasmic membranes. Antibodies against the H and F proteins stained very similarly; primarily a trabeculated (small rod) membrane staining as well as granular cytoplasmic staining especially in syncytia. In a panel of eleven different monoclonal antibodies directed against the F

protein, three were found to cross-react with a 79,000 MW cellular stress protein located primarily in the cytoplasm (Sheshberadaran and Norrby, 1984). Stress proteins are produced by cells in response to stresses such as virus infection and heat shock.

Norrby et al. (1982) examined MV lytically infected cells and MV persistently-infected Lu 106 cells with monoclonal antibodies and found that all persistently-infected cells produced NP and P antigens but had a restricted capacity to produce detectable amounts of envelope antigens. Approximately 50% of the cells stained weakly to strongly with H, 5% stained weakly with F, and 25% stained weakly to moderately with M antibody. Norrby et al. (1982) proposed that a restricted capacity to produce biologically active F protein was necessary for maintaining a persistent infection in actively dividing cells in vitro.

A reduced amount of M, H and F antigens was also observed in cells infected with three strains of SSPE viruses (Biken, IP-3 and DR) compared to cells infected with wild type MV (Edmonston) and the Lec SSPE strain (Johnson et al., 1982). All of the strains produced large amounts of NP and P as detected by immunofluorescence. Edmonston and Lec infected cells stained brightly for M, H and F antigens and produced high titers of infectious virus. The Biken strain produced moderate titers of extracellular virus and moderate amounts of M, H and F antigens whereas the DR strain produced no infectious virus and lacked detectable M or F but had trace amounts of H antigen. The IP-3 strain produced very few infectious particles and had no detectable M antigen but moderate levels of H and F antigens. The expression of MV antigens was variable and seemed to be related to the titer of virus produced (Johnson et al., 1982).

Monoclonal antibodies were used to study the cell-associated SSPE cell line characterized by limited syncytia development and plaque healing (Norrby et al., 1985). Pronounced staining of the syncytia was obtained with NP and P monoclonal antibodies and intranuclear inclusions contained NP antigen. Staining with monoclonals against H and F proteins resulted in weak staining and one of four anti-F antibodies gave no staining. M protein appeared to be absent because numerous monoclonal antibodies (representing all six

epitopes) failed to stain the syncytia. No mention was made about the immunofluorescence status of the cells which were filling in the plaques.

Differences in the accumulation and antigenic character of various MV proteins in lytically and persistently-infected cells, both infected with the same strain of MV, have been described (ter Meulen et al., 1981; Giraudon, Gerald and Wild, 1984). Giraudon et al. (1984) compared the distribution of virus antigens by immunofluorescence in Halle infected African green monkey kidney (BGM) cells, both in a persistently-infected cell line and in acutely infected cells. A major difference was that up to 50% of the persistent cells had intranuclear dots containing NP antigen as compared to 5% in the lytic infection and the quantity of L protein was greatly reduced relative to the other viral proteins in these persistently-infected cells. This protein was at the limit of detection by the 273rd passage level. The quantity of NP, P, M and H protein was constant during persistence. ter Meulen et al. (1981) discovered that the hemagglutinin protein of the Lec SSPE isolate changed antigenically during the course of a persistent infection. Six different anti-H monoclonal antibodies stained cells lytically infected with the Edmonston or Lec strain whereas only two of the clones gave positive immunofluorescence against the persistent infection, supporting the observation that mutations arise during viral persistence.

1.5.2 Ultrastructural Studies

Measles virus infected cells have been studied at the ultrastructural level with the electron microscope. Shortly after the isolation of MV in tissue culture in 1954, and the observation of inclusion bodies by light microscopy, the fine structure of these cellular inclusions was studied by electron microscopy (Kallman et al., 1959). They observed randomly arrayed filaments of low electron density in the cytoplasm and nucleus of Edmonston infected HeLa cells. Dense, highly ordered arrays of filaments were seen in the nuclei as well. The cytoplasmic helical nucleocapsid aligned under the cell membrane, which had been modified to include an outer layer of short projections, and was packaged into virus particles by a budding process (Nakai and Imagawa, 1969; Nakai, Shand and Howatson, 1969;

Rain et al., 1969). The cytoplasmic nucleocapsids consisted of fine (20 nm) tubules, each surrounded by an outer rim of less-electron-dense material with a total diameter of 40 to 50 nm. The intranuclear nucleocapsids lacked this outer layer of fuzzy material and were called "smooth" nucleocapsids (Raine et al., 1969; Fournier, Privat and Bouteille, 1981), whereas the cytoplasmic ones were called "fuzzy", "rough" or "granular" nucleocapsids. This fuzzy material is thought to be the MV M protein which plays a role in the assembly of the budding virus at the plasma membrane.

Smooth nucleocapsids have also been found in the cytoplasm of SSPE nonproductive cells. They have been seen in SSPE brain specimens (Dubois-Dalcq, Coblentz and Pleet, 1974), SSPE brain cell cultures (Oyanagi, et al., 1970) and SSPE latently infected cell lines (Doi et al., 1972; Dubois-Dalcq et al., 1974; Sheppard et al., 1975). This aberrant form of cytoplasmic nucleocapsid appears to be related to the failure of the virus infection to assemble virions (as they are not found in productive SSPE virus infections).

The usual form of cytoplasmic nucleocapsid is the fuzzy type. Cytoplasmic nucleocapsid inclusions are found in both acute and persistent MV infections, but nuclear inclusions are found much more frequently in persistent infections (Oyanagi et al., 1970, 1971; Robbins and Eagle, 1985). Nakai, Shand and Howatson (1969) reported that cytoplasmic nucleocapsid formation was associated with active budding and high virus yields, whereas when intranuclear nucleocapsid formation was predominant, little or no budding was detected and virus yields were low. Nonproductive MV or SSPE virus infected cells are characterized by a lack of nucleocapsid alignment under the cell membrane and no budding viral particles as seen by electron microscopy (Dubois-Dalcq et al., 1974).

The presence of smooth nucleocapsid in the nucleus of MV infected cells has been well-documented from the time of the early electron microscopic examinations of SSPE brain material (Perier, Vanderhaeghen and Pelc, 1967) to the present time (Robbins and Eagle, 1985). The function of this nucleocapsid material is unknown, but it is not thought to be involved in virion production. Electron microscopic examinations of the nuclear envelope have not shown obvious changes or defects to suggest a possible exchange of nucleocapsids between

the cytoplasm and nucleus (Fournier, Privat and Bouteille, 1981).

Another feature that has been observed in the nuclei of SSPE brain specimens (Perier et al., 1967), MV-infected and SSPE virus-infected cells is the ocurrence of simple and complex nuclear bodies (NB; Fournier et al., 1981; Nakai, Shand and Howatson, 1969; Raine et al., 1969, 1974; Dubois-Dalcq et al., 1974; Robbins and Eagle, 1985; Oyanagi et al., 1970). Simple NB appear as pale, round or oval structures sometimes having a complex lamellar pattern (De Thè, Riviére and Bernhard, 1961; Hinglais-Guillaud, Moricard and Bernhard, 1961). It is now known that simple NB are proteinaceous structures which exist under normal circumstances in many, if not all, types of cells (Bouteille, Laval and Dupuy-Coin, 1974). The function of the NB is unknown, but they are more numerous in hyperactive cells, eg., cells stimulated by physiological, hormonal, drug-induced, viral or tumoral factors (Bouteille, Kalifat and Delarue, 1967). Oyanagi et al. (1970) hypothesized that NB could be early foci in the formation of NC which is supported by the fact that viral NC material has been seen to be associated with NB (Oyanagi et al., 1970; Fournier et al., 1981). Complex NB are divided into the granular and beaded types and contain granules of ribonucleprotein surrounded by a fibrillar proteinaceous capsule. The relationship of complex NB to the simple NB is unclear and they are not found in normal tissue (Dupuy-Coin and Bouteille, 1972).

Nuclear bodies have been reported by a number of investigators working with MV, SSPE virus and SSPE brain material. At least one NB was found in 33.5% of the nuclei in SSPE brain material; 90% were of the simple type and 10% were the complex type (Dupuy-Coin and Bouteille, 1972). Cell cultures from SSPE biopsy material contained complex NB in the early passages and bundles of nucleocapsids inside fibrillar rings (viral NB) were seen rarely (Oyanagi et al., 1970). Complex NB and nucleocapsid material were often seen within a common area of the lymph node nuclei in Rinderpest virus infected cattle (Tajima and Ushijima, 1971). Nuclear bodies were commonly seen in Edmonston infected hamster cerebellar explants (Fournier et al., 1981). At twenty days after infection 100% of the cells contained simple NB and at thirty-two days following infection, 46% contained complex NB and 2% had viral NB. Fournier et al. (1981) postulated that the NB were acting as

specific target organelles for viral infection and/or the possible sites for the assembly of nucleocapsids, proteins and MV RNA. Nuclear bodies present in virus-infected cells may be markers for persistent infections both in vivo and in vitro.

1.6 Current Research Project

The aim of this project was to follow the temperature-dependent production of a persistent infection of Vero cells by the Halle strain (SSPE isolate) of MV using the techniques of immunofluorescence and electron microscopy. As previously mentioned, the Halle strain displays a temperature-dependent production of interfering virus particles (Tsang et al., 1981) which are believed to be responsible for the establishment of this persistent infection. Using monoclonal antibodies directed against five structural proteins (NP, P, M, H and F) of MV, the persistently (39°C) and lytically (32°C) infected cultures were examined by immunofluorescence to determine possible differences (ie. protein accumulation) at the two temperatures. The cultures were also studied by electron microscopy in order to detect differences at the ultrastructural level. In order to become very familiar with the immunofluorescent stained appearances of the five viral proteins in a normal lytic infection, a detailed time course study was undertaken so as to follow the changing intracellular amounts and distributions of the specific MV components over time. Such a detailed IF study of the development of a MV infection in cell culture had not previously been reported.

2. Methods and Materials

2.1 Tissue Culture

Vero (African green monkey, Cercopithecus aethiops, kidney; ATCC CCL 81; obtained from Flow Laboratories, Inglewood, California) cells were used throughout this research. The cells were grown in glass culture bottles (Blake bottles) at 37°C and were passaged approximately every 7 days using the following procedure: the tissue culture medium was removed and the cell monolayer rinsed with 37°C phosphate buffered saline (PBS; see appendix). Two mL of 0.25% (w/v) trypsin (Difco Laboratories, Detroit, Michigan) in PBS was added to the Blake bottle. Incubation at 37°C continued until all cells had detached from the glass surface. Vigorous pipetting was used to suspend the cells in 10 mL of growth medium. Aliquots of 4 mL containing approximately 1.5 X 10^7 cells (as determined by a hemacytometer count) were added to each of 3 Blake bottles containing 125 mL of growth medium.

Cells for infection were grown in 50mm diameter tissue culture dishes (Corning Glass Works, Corning, New York), 25 cm^2 or 75 cm^2 tissue culture flasks (Corning). Mycoplasma culture tests were done at regular intervals on these cells (by Dr. J. Robertson, University of Alberta, Edmonton, Alberta) and the results were negative.

2.2 Tissue Culture Medium

Cell growth medium was prepared from powdered Eagle's modified minimal essential medium (MEM) lacking glutamine and sodium bicarbonate (Auto-Pow medium, Flow Laboratories). The powder was dissolved in glass-distilled water and dispensed into glass bottles. The media was sterilized by autoclaving at 120°C and 15 psi for 20 mins. Before use, 10 mL of heat-inactivated bovine calf serum (Gibco Laboratories, Grand Island, New York; 2% final concentration), 5 mL of a solution containing 10,000 IU/mL penicillin G sodium (100 IU/mL, final concentration) and 10 mg/mL streptomycin sulphate (0.1 mg/mL, final concentration), 5 mL of a 0.2 M glutamine solution (0.002 M, final concentration), and 6 to

10 mL of a 7.5% (w/v) sodium bicarbonate solution were added to each 475 mL of medium. The sodium bicarbonate and glutamine solutions were filter sterilized (0.22 micrometer pore size, Millipore Ltd., Mississauga, Ontario). The antibiotic solution was prepared using sterile antibiotics and sterile water.

2.3 Virus

The Halle strain of measles virus (MV-H; ATCC VR-804) was a gift from Dr. T. F. Wild (I.N.S.E.R.M., Lyon, France). This strain was originally isolated from the lymph node biopsy of a 12 year old female with clinical SSPE symptoms (Horta-Barbosa et al., 1971). The MV-H virus strain was plaque-purified in this laboratory. Virus obtained from distinct "ring plaques" (Fig. 2) grown at 37°C was further plaque-purified and designated as ring plaque (RP) derived virus.

The Lec strain of measles virus (MV-L), originally isolated from an SSPE patient, was obtained as a gift from Dr. E. Norrby (Karolinska Institute, Stockholm, Sweden). Two times plaque-purified virus was used to produce the IM stock.

Mumps virus used in this study was a wild-type isolate from a patient having typical mumps in Finland and was a gift from Dr. A. Salmi (University of Alberta).

2.4 Virus Propagation

Cell monolayers were infected at 70-100% confluency. Following removal of the tissue culture medium, infectious virus was added directly to the monolayer. Virus was adsorbed for 1 hour at 37°C and new growth medium was added. The MV-H strain was grown at 32°C, 37°C, or 39°C, the MV-L strain was grown at 32°C and 39°C and mumps virus was grown at 32°C and 37°C.

Serial undiluted passage of Halle strain at 37°C or 39°C induced the production of defective-interfering (DI) particles (Tsang et al., 1981). In this study, the first undiluted passage of virus was referred to as "UP-1". When UP-1 was used in undiluted form to infect cells, this was called "UP-2", etc.

2.5 Plaque Assay

Determination of infectivity and plaque morphology were carried out by standard plaque assay methods (Rovozzo and Burke, 1973). Tissue culture dishes were seeded with 2 X 10^6 Vero cells and incubated overnight at 37°C in a 5% CO_2 atmosphere. Dishes were infected the next day at 70-100% confluency. Duplicate dishes were infected with 0.1mL of 10-fold viral dilutions. After adsorption at 37°C for 1 hour, 10 mL of overlay medium was added to each dish. The overlay medium consisted of 5 mL of 2% special agar-noble (Difco Laboratories, Detroit, Michigan; 1% (w/v) final concentration) and 5 mL of two-times concentrated Eagle's modified MEM lacking glutamine, phenol red, and sodium bicarbonate (Flow Laboratories) supplemented with two-times the normal media concentrations of calf serum, penicillin, streptomycin, glutamine and sodium bicarbonate. Before addition to the monolayers, the overlay medium was cooled to 45-50°C to prevent heat shock to the cell monolayer.

The infected cell dishes were incubated at 32°C, 37°C, or 39°C in a 5% CO_2 atmosphere at 90% relative humidity. The cells were fixed in 15% acetic acid at 5-14 days post infection. Fixation was performed for 3 hours or overnight. The plates were washed with water and the agar overlay carefully removed with a spatula. A solution of crystal violet (see appendix) was used to stain the monolayers. Following numerous water rinses, the plates were air dried and the plaques counted.

2.6 Hemadsorption

Hemadsorption (HAD) was performed on infected and non-infected Vero cell monolayers. A 1% v/v suspension of Cercopithecus aethiops monkey erythrocytes (Connaught Laboratories Ltd., Willowdale, Ontario) in PBS was added to cell monolayers which had been rinsed twice with 37°C PBS. Following a 10-15 minute incubation at 37°C, the excess erythrocytes were removed by rinsing three times with warm PBS. The results were observed using an inverted light microscope.

2.7 Immunofluorescence

Immunofluorescence (IF) assays were performed on Vero cell monolayers grown on 10.5 X 35 mm glass coverslips (Bellco Glass Inc., Vineland, New Jersey). In preparation for cell growth, the coverslips were washed in a solution of Haemo-Sol (Haemo-Sol Inc., Baltimore, Maryland) and rinsed well in tap water followed by a deionized water wash. The clean coverslips were stored in 95% ethanol until needed, at which time each was flamed and two coverslips placed into a 60 mm diameter tissue culture dish. The culture dishes were seeded with 2 X 10^6 cells and the monolayers infected the next day with 0.2 mL of virus preparation. Five mL of fresh growth medium was added after adsorption. The dishes were incubated at 32°C, 37°C, or 39°C in a 5% CO_2 atmosphere. Coverslips were removed from the culture dishes at selected times post-infection, rinsed with PBS, and fixed at room temperature in cold acetone (-20°C) for 10 min. The coverslips were air dried and stored in sealed tubes at -70°C until stained.

The immunofluorescent staining procedure made use of a fluorescent antibody staining system (Bellco Glass) consisting of staining trays and a humidity chamber. Coverslips wetted with PBS were set inverted over a trough in the staining tray, and small volumes of antisera were added to the edge of the coverslip and were drawn under the coverslip by capillary action.

The primary antisera used were mouse monoclonal antibodies (supplied by Dr. E. Norrby and Dr. T. F. Wild) prepared against selected MV components, a monoclonal antibody specific for the P protein of CDV and rabbit polyclonal antiserum (supplied by Dr. A. Salmi) prepared against wild-type MV and MV NC material. Table 1 lists the specificities, designations, and working dilutions of each antiserum used.

The staining trays holding the coverslips and primary antiserum were incubated in a humidity chamber either at 37°C for 30 min or at room temperature for 2 - 3 hours. Following primary antibody staining, the coverslips were washed by repeated dipping in three changes of PBS. The coverslips were then placed onto clean staining trays and secondary antibody added. In the case of monoclonal antibody staining, the secondary antibody was a

Table 1.

Antisera used for immunofluorescence assays

Source	Specificity	Designation	Working Dilution
Norrby	NP (Lec)	16AC5	1:100
	P (Lec)	16AF10	1:50
	P (Lec)	16BD3	1:50
	M (Lec)	19CG6	1:20
	M (Lec)	19DC5	1:20
	H (Lec)	16DE6	1:100
	H (Lec)	16CD11	1:100
	F (Lec)	19BG4	1:50
Wild	P (Halle)	C1-105	1:200
	M (Halle)	C1-144	1:100
	H (Halle)	C1-15	1:150
Salmi	Polyclonal (wild-type)	MVR-3	1:200
	Polyclonal (nucleocapsid)	MeNC-1	1:200
	CDV-P	X / 32	1:100

1:30 dilution of goat-anti-mouse antibody conjugated to fluorescein isothiocyanate (GAM-FITC, Nordic Immunological Laboratories, Tilburg, The Netherlands). Goat-anti-rabbit antibody conjugated to FITC (GAR-FITC, Nordic Immunological Laboratories) was used to stain the rabbit polyclonal antiserum. Incubation of the secondary antiserum was carried out in the same way as the primary antiserum. The coverslips were washed by repeated dipping into three changes of PBS and mounted, cell-side-down, on glass slides with buffered glycerin (9:1, glycerin:PBS). The results were observed with a Leitz Dialux 20 microscope equipped for incident light fluorescence. The light source was a super pressure mercury lamp (HBO 50W) supplied by Osram (Berlin, Germany). The Leitz filter block (I2) was composed of the following: exciting filter (450-490 nm), beam splitting mirror (510 nm) and suppression filter (515 nm).

2.8 Preparation of Samples for Electron Microscopy

MV-infected cells were examined using transmission electron microscopy. Cells were processed and embedded using the cell pellet method. Cell monolayers infected with MV were rinsed with phosphate buffer followed by a rinse with cold 3.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (see appendix). Fixation was carried out with 3.5% glutaraldehyde for 20-30 min. The cells were gently scraped off the plastic surface using a rubber policeman and pelleted in a conical centrifuge tube for 20 min at 2500 g to form a hard pellet. The glutaraldehyde fixative was discarded and the cell pellet rinsed gently with 7% (w/v) sucrose in 0.1 M phosphate buffer. Following this step, the cell pellet was stored overnight at 4°C or the fixation and dehydration continued. The pellet was transferred to a disposable glass vial and postfixed for 1 hour in 1% (w/v) OsO_4 in 0.1 M phosphate buffer. Following the removal of the OsO_4 solution, the cell pellet was rinsed with 50% (v/v) ethanol and fresh 50% ethanol added for 15 min. Dehydration was carried out as follows: two 15 min intervals in 75% ethanol, two 15 min intervals in 90% ethanol, two 30 min intervals in 100% ethanol, and two 30 min intervals in propylene oxide (Fisher Scientific, Edmonton). The cell pellets were left overnight in a 1:1 mixture of propylene oxide and LX-112 embedding resin (Ladd

Research Industries, Burlington, Vermont; see appendix). The next day, the pellets were cut into smaller pieces and added to flat molds pre-filled with LX-112 resin. Polymerization of the resin took place at 60°C for 3 days.

2.9 Ultramicrotomy and Electron Microscopy

Silver to gold sections (60-100 nm thickness) were cut using a diamond knife (Dupont Instruments, I & B/Maynard Scientific, Mississauga, Ontario) or glass knives (LKB Glass, Bromma, Sweden) made on a LKB knifemaker (model 7801B). Sections were mounted on formvar-coated 200 mesh copper grids and stained with 5% (w/v) uranyl acetate (Fisher Scientific Co.) in 100% methanol for 10 min followed by a water wash and drying. The grids were floated on drops of lead citrate (see appendix) for 2 to 4 mins, rinsed in water and dried prior to examination.

All grids were examined in a Philips EM 300 electron microscope operating at 80 kV.

2.10 Photography

Photographs recording the CPE and HAD of MV infected cells were taken using an Olympus inverted light microscope (Model IMT-213) equipped with an Olympus photomicrographic camera system. The film used was Kodak Tri-X Pan (DIN 27; Kodak Canada, Toronto, Ontario).

Immunofluorescence photographs were taken using a Leitz Dialux 20 incident light fluorescent microscope and Wild photomicrographic system (Photoautomat MPS 45/51S). Kodak Tri-X Pan film was used.

All films were developed using Kodak chemicals and printed on Kodak polycontrast rapid II, glossy, resin-coated paper. The enlarger used was a Durst Laborator model 138 S (Bolzano, Italy). Prints were processed using a Kodak Ektamatic processor and dried with a Ilfospeed 5250 print drier.

2.11 Thesis Preparation

This thesis was prepared in part using the University of Alberta word processing program "Textform" operating on an Amdahl 5860 computer and supporting the Michigan Terminal operating System (MTS). Text printing, using the Press-Roman font, was done by a Xerox X9700 Laser Printer. Tables and schematic figures were prepared with an Apple MacIntosh microcomputer and Apple LaserWriter Printer using the Times font.

3. Results

3.1 Growth Characteristics of MV-Halle

The Halle strain of MV produced DI particles at 37°C and 39°C and the cyclic changes in the amounts of DI and infectious virus were graphically illustrated by the "ring plaques" produced at 37°C (Fig. 2a). These ring plaques were not formed at 32°C or 39°C as illustrated in a plaque assay of 32°C prepared Halle IM assayed at 32°C and 37°C (Fig. 2b). Different results were obtained when the Halle IM was prepared at 37°C. This material produced clear (lysed) plaques at 32°C and non-clear (unlysed), non-ring plaques at 37°C (not shown).

Undiluted passage of Halle IM at 39°C led to a decreased production of infectious virus and the temperature-dependent production of DI particles. Figure 3 schematically illustrates the results and experimental design of undiluted passage at 32°C and 39°C. Halle IM prepared at 32°C was used in undiluted form to infect cells at an multiplicity of infection (moi) of approximately 4 pfu/cell (Fig. 3). The UP-1 culture at 32°C (UP-1(32)) was totally destroyed by 3 d pi with a resulting virus titer of $5.5X10^7$ pfu/mL. In contrast, the UP-1 culture at 39°C (UP-1(39)) which was also destroyed had a much lower titer of infectious virus ($5.9X10^3$ pfu/mL), a 10,000 fold reduction of extracellular virions. The UP-1(39) culture medium was used in undiluted form to infect cells at an moi of 0.001 pfu/cell. The UP-2 culture at 32°C (UP-2(32)) developed 4+ CPE by 7 d pi and produced high levels of infectious virus ($3.4X10^7$ pfu/mL). The CPE did not progress in the UP-2 culture at 39°C (UP-2(39)) and was characterized by the "healing syncytium" phenomenon. Syncytia were seen to detach from the substrate, leaving a hole or plaque in the monolayer which subsequently was filled by the inward growth and multiplication of neighbouring cells. Infectious virus could not be deteced in these UP-2(39) cultures and the infected cells could be passaged. Passaged cells incubated at 32°C produced 4+ CPE by 10 days post passage (d pp) and infectious virus was produced (10^5-10^6 pfu/mL). The passaged cells at 39°C resembled the UP-2(39) culture with respect to the healing syncytia and lack of infectious virus produced.

3.2 Study of Halle (Ring Plaque) Grown at 32°C

The purpose of this experiment was to study the course of a "normal" infection with MV-H during a four day period. At selected times post-infection, samples were examined for CPE, HAD, virus production, immunofluorescent staining, and selected samples were examined at the electron microscope level.

Vero cells grown in 50mm tissue culture dishes were infected with RP-derived virus (prepared at 32°C) at a moi of 0.02 pfu/cell and incubated at 32°C. Samples of infected cells were taken at 1 h intervals for the first 24 h period post-infection, at 2 h intervals for the next 24 h period and at 4 h intervals for the following 48 h period (total time of infection was 96 h). Samples of mock-infected, negative control Vero cells were taken at 8 h intervals throughout the 96 h period. Each sample was photographed to record the amount of CPE and HAD. A sample of the tissue culture medium was also taken at each time and stored at -20°C until plaque assayed at 32°C to determine the amount of extracellular infectious virus. As well, coverslips of each sample were fixed and stored at -70°C until needed for immunofluorescent assays using the following MV monoclonal antibodies: NP (16AC5), P (Cl-105), M (Cl-144), H (16DE6) and F (19BG4). Samples were prepared for electron microscopic observation by the cell pellet method.

3.2.1 Development of Cytopathic Effect and Hemadsorption

CPE in the form of syncytia (multi-nucleated cells; Fig. 4) was clearly seen 40 h pi. At 36 and 38 h pi, small areas were observed which may have been the early stage of syncytia development. Fifty percent of the monolayer showed syncytia formation (cell fusion) by 60 h pi and at 80 h pi 75% of the monolayer was involved in cell fusion (Table 2). At the end of the incubation period (96 h) less than 100% of the cells were showing CPE as "normal looking" cells could be found (Fig. 4g).

Weak HAD was observed on syncytia at 44 h pi (Fig. 4b; Table 2) and it was not until 52 h pi that substantial numbers of erythrocytes attached to syncytial surfaces such that 25 - 50% of the syncytial area had adsorbed erythrocytes (2+). At 72 h pi, approximately 50

Table 2.

Immunofluorescent staining of viral antigens and progression of CPE and HAD in the study of RP at 32°C

Hours	CPE	HAD	NP	P	M	H	F
6	-	-	1+ §	-	-	-	-
7	-	-	1+	-	-	-	-
8	-	-	1+	-	-	-	-
9	-	-	1+	-	-	-	-
10	-	-	1+	1+	-	-	-
11	-	-	1+	1+	-	-	-
12	-	-	1+	1+	-	-	-
13	-	-	1+	1+	-	-	-
14	-	-	1+	1+	-	-	-
15	-	-	1+	1+	-	-	-
16	-	-	1+	1+	-	-	-
17	-	-	1+	1+	-	-	-
18	-	-	1+	1+	1+	-	-
19	-	-	1+	1+	1+	-	-
20	-	-	1+	1+	1+	1+	-
21	-	-	1+	1+	1+	1+	1+
40	1+ §	-	1+	1+	1+	1+	1+
44	1+	1+ †	2+	2+	2+	2+	1+
48	1+	1+	2+	2+	2+	2+	2+
60	2+	2+	3+	3+	3+	3+	3+
72	3+	3+	4+	4+	4+	4+	4+
96	4+	4+	4+	4+	4+	4+	4+

§ Estimation of the amount of CPE or IF: 1+, less than 25% involvement; 2+, 25% - 50%; 3+, 50% - 75%; 4+, 75% - 100%.

† Estimation of the amount of HAD: 1+, less than 25% of syncytial area; 2+, 25% - 50%; 3+, 50% - 75%; 4+, 75% - 100%.

75% of the fused cell area was covered with erythrocytes (3+; Fig. 4f) and almost 100% of the syncytial area had attached erythrocytes at 96 h pi (Fig. 4h).

3.2.2 Virus Production

The production of extracellular virus was first detected at 36 h pi (1.5×10^2 pfu/mL). At this time the infectious virus titer was increasing over the background level of infectious virus. Rinsing the cells after virus adsorption did not eliminate all the infectious inoculum virus as shown by the virus titers between the times of 24 to 34 h pi (Table 3; Fig. 5). The virus titer increased over the duration of the experiment to a value of 2.2×10^6 pfu/mL at 96 h pi.

3.2.3 Immunofluorescent Staining

The staining patterns observed for the NP and P protein antigens were very similar. Figure 6 is a schematic representation of the progression of the NP and P immunofluorescent staining. As well, the H and F proteins stained similarly (Fig. 8) but the M protein staining (Fig. 7) was different from the other four proteins.

3.2.3.1 NP Protein

The NP protein was detected as early as 6 h pi in the form of tiny fluorescing cytoplasmic dots (Fig. 9a). These dots increased in size such that single cells contained dots of various sizes (Fig. 9b) and nuclei were nonstaining. Twenty-two hours after infection, cells were also beginning to show a flecked (small rod; Fig. 9c) type of staining throughout the cytoplasm. As time progressed, the dot-like inclusions became larger, irregular globular inclusions and the flecks aggregated into fibrous material which was observed to loosely pack into fibrous inclusions. The typical large syncytia at 48 or 72 h pi was characterized by a central area which stained with the fibrous type of staining and a few fibrous inclusions. Bright globular inclusions were found adjacent to the syncytial nuclei and the flecks and brightly fluorescing dots of various sizes were found within the outer boundaries of the syncytia (Fig. 9d,9e). By 88 h pi, all cells were positively stained

Table 3.

Production of extracellular virus by RP at 32°C

H.P.I.	PFU/ML
24	8.5×10^1
26	6.5×10^1
28	6.0×10^1
30	7.0×10^1
32	7.0×10^1
34	9.0×10^1
36	1.5×10^2
38	7.1×10^2
40	3.6×10^2
42	7.0×10^2
44	1.2×10^3
46	9.0×10^2
48	2.6×10^3
52	1.2×10^4
60	2.6×10^4
64	4.2×10^4
68	1.2×10^5
72	1.7×10^5
76	3.2×10^5
80	2.4×10^5
84	5.5×10^5
88	1.8×10^6
96	2.2×10^6

by the anti-NP monoclonal antibody.

3.2.3.2 P Protein

The immunofluorescent staining appearance of P protein was indistinguishable from the NP protein pattern. Tiny cytoplasmic dots of P protein were first detected at 10 h pi (Fig. 10a) and at 19 h pi a variety of dot sizes was seen. Flecking was first observed at 20 h pi and is illustrated in figure 10b. A syncytia photographed at 48 h pi (Fig. 10c) shows the typical bright dots and globules as well as flecks and fibrous type staining. Each of these staining types and fibrous inclusions was evident in a large syncytia at 72 h pi (Fig. 10d). All cells were fluorescing by 88 h pi.

3.2.3.3 M Protein

The pattern of M protein staining was distinct from that obtained with the other four proteins because of the nuclear staining and two kinds of syncytial staining. Single cells showing a fine homogeneous cytoplasmic staining, a few pale cytoplasmic globules, and diffuse nuclear staining were observed as early as 18 h pi (Fig. 11a) and 24 h pi (Fig. 11b). Staining of the nucleoplasm appeared to spare the nucleoli and was a similar intensity as the homogeneous cytoplasmic staining. Staining of the cytoplasm progressed to flecks and fibrous type material along with a few globules (48 h pi; Fig. 11c). After 40 h pi, two types of syncytia with very different stained appearances were observed. Most commonly seen was the brightly staining, flecked and fibrous type with diffuse nuclear staining (Fig. 11d). The less frequently seen type was characterized by a lack of flecks and fibrous material, characterized by weakly stained homogeneous cytoplasm containing pale globular perinuclear inclusions and diffuse nuclear staining (Fig. 11e). Occasionally large syncytia were observed which were nonstaining by anti-M monoclonal antibody. All cells except for the nonstaining syncytia, were positively stained by 88 h pi.

3.2.3.4 H Protein

H protein was detectable by 20 h pi as a pale homogeneous cytoplasmic staining with a few flecks (Fig. 12a). The clarity and fluorescent intensity of the flecking

increased over time (24 h pi; Fig. 12b). Distinct flecking and fibrous type staining was evident at 40 h pi (Fig. 12c). The fibrous material formed fibrous clusters which appeared to be at the cell surface (Fig. 12d). By 88 h pi, all cells were staining for the H protein.

3.2.3.5 F Protein

Twenty-one h pi, F protein was detected as pale cytoplasmic flecks (Fig. 13a). The intensity of the flecks was dull at 24 h pi (Fig. 13b) but increased with increasing time post infection. Small syncytia at 40 h pi showed fibrous material whereas single cells were only flecked. Intense flecking, fibrous material and fibrous clusters were observed at 48 h pi (Fig. 13c) and 72 h pi (Fig. 13d). The entire monolayer was fluorescing at 88 h pi.

3.2.4 Electron Microscopic Observations

Samples taken at 72 and 96 h pi were examined using the electron microscope. Nucleocapsid tubules forming cytoplasmic inclusions were observed at 72 h pi and in greater than 50% of the cells at 96 h pi (Fig. 14a). These fuzzy NC had a diameter of approximately 39 nm. Modified regions of the plasma membrane were observed which had a thickened and fuzzy appearance (Fig. 14b, 14c). These modifications are associated with viral budding. Extracellular virus particles were observed at 72 h pi (Fig. 14b) and 96 h pi. Occasionally the nuclei of infected cells at 96 h pi contained fibrillar simple nuclear bodies (simple NB; Fig. 14c).

3.3 MV-Lec and Mumps Virus-Infected Cells

Vero cells grown on coverslips were infected with the Lec strain of MV and mumps virus to determine the IF staining pattern with the anti-M monoclonal antibody (C1-144).

MV-L infectious material was used to infect cells at multiplicities of 0.5 pfu/cell and 0.005 pfu/cell. The infected coverslips were incubated at 32°C and 39°C and coverslips were fixed in acetone when syncytia were evident. IF assays were done using the C1-144, 19CG6

and 19 DC5 clones of anti-M monoclonal antibodies.

Mumps virus at approximately 0.001 pfu/cell was used to infect cells on coverslips. Following infection, the cells were incubated at 32°C and 37°C plus uninfected control coverslips at 37°C were included. Infected cells were fixed 4 and 5 d pi at which time 2+ CPE or greater was observed. An IF assay using the monoclonal antibody C1-144 clone was performed.

Diffuse nuclear staining like that seen in Halle-infected cells was also observed in Lec-infected cells stained with the C1-144 clone of anti-M monoclonal antibody (Fig. 15a). This type of nuclear staining was clearly seen at 39°C (both 0.5 and 0.005 pfu/cell) and less clearly at 32°C. The monoclonal antibody clones 19CG6 and 19DC5 stained only cytoplasmic material. The intensity and amount of IF staining at both temperatures was two to three times greater at the lower moi (0.005 pfu/cell).

The nuclei of mumps virus syncytia at 32°C and 37°C did not display the characteristic diffuse nuclear staining seen with Halle and Lec-infected cells following staining with the C1-144 clone (Fig. 15c). The nuclei present within syncytia stain in the same manner as the nuclei of non-fused neighbouring cells or uninfected control cells (Fig. 15d).

3.4 Halle Undiluted Passage-1 Study

Halle IM prepared at 37°C ($4.5X10^5$ pfu/ml) was used in undiluted form to infect tissue culture dishes containing two coverslips at a moi of approximately 0.02 pfu/cell. Infected coverslips at 39°C were fixed for IF assay at 8, 18, 27 and 42 h pi and 4 d pi. Infected coverslips at 32°C were fixed at 51 h pi and 4 d pi. Uninfected control coverslips at both temperatures were included in IF assays. The following antisera were used for IF staining: polyclonal wild-type (MVR-3), polyclonal NC (MeNC-1), NP (16AC5), P (C1-105, 16AF10, 16BD3), CDV-P (X/32), M (C1-144), H (C1-15, 16DE6, 16 CD11) and F (19BG4). HAD tests were performed on the 42 h pi sample at 39°C and the 51 h pi sample at 32°C. Infected cells at 39°C showing 4+ CPE at 3 d pi were fixed and embedded for electron microscopy as were infected cells at 32°C demonstrating 4+ CPE at 5 d pi. Negative controls at both temperatures were also prepared for electron microscopy.

3.4.1 Development of Cytopathic Effect and Hemadsorption

CPE consisted of the formation of syncytia at 39°C and 32°C. No syncytia were observed at 8 or 18 h pi at 39°C although 1+ CPE was observed 27 h pi and the CPE had reached 3+ by 42 h pi and 3+ to 4+ at 3 d pi at 39°C. Infection at 32°C proceeded more slowly. At 51 h pi, the infected cells at 32°C demonstrated 1+ CPE and at 4 d pi 3+ to 4+ CPE was observed.

The amount of HAD at 32°C (51 h pi) was found to vary depending on the individual syncytium from 1+ to negative HAD. Likewise, the syncytia at 39°C were found to vary from 1+ to 3+ HAD per individual syncytium.

3.4.2 Immunofluorescent Staining

Differences were observed in the IF staining patterns obtained at 32°C and 39°C. Direct comparison of IF staining was made 51 h pi with samples maintained at 32°C and 27 h pi with samples maintained at 39°C when both infected cultures displayed 1+ CPE. The most noticeable differences were seen in the M, H and F protein staining, with smaller differences noted for the NP and P proteins (Table 5). Table 4 explains the notation representing the different types of IF staining.

3.4.2.1 Polyclonal Wild-Type Antiserum

Polyclonal wild-type antiserum stained both 32°C and 39°C syncytia intensely (Fig. 16a, 16b). The 32°C syncytial cytoplasm contained dots and globules, flecks, fibrous material, fibrous inclusions and an overall pale homogeneous staining (Table 5). The 32°C nuclei had a pale diffuse fluorescence which was not as bright or distinctive as the nuclear staining obtained with the polyclonal NC (Fig. 17a) or M protein monoclonal antisera (Fig. 22a). Occasionally a nucleus could be seen at 32°C which contained bright fluorescent dots (type 7n). The 39°C syncytia at 37 h pi stained similarily although not as highly flecked as 32°C syncytia and generally lacked the fibrous inclusions. No nuclear dots were observed 27 h pi at 39°C but these were commonly found at 42 h pi along with the various types of cytoplasmic staining (Table 5). The majority of the cytoplasmic

Table 4.

Immunofluorescent staining types

Cytoplasmic	
Type 1	Bright dots and/or bright globules
Type 2	Flecks
Type 3	Fibrous
Type 4	Fibrous inclusion
Type 5	Fibrous clusters
Type 6	Fine homogeneous
Nuclear	
Type 7 n	Bright dots
Type 8 n	Diffuse nuclear staining around nucleoli

Table 5

Halle UP-1 immunofluorescent staining

Antiserum	32°C 51 hpi	39°C 27 hpi	39°C 42 hpi
Polyclonal wt (MVR-3)	**1,2,3,4,6,8n** 7n	**1,2,3,6,8n**	**1,2,3,4,6,7n,8n**
Polyclonal NC (MeNC-1)	**1,2,3,4,6,8n** 7n	**1,2,3,6,8n** 4,7n	**1,2,3,4,6,7n,8n**
NP (16AC5)	**1,2,3,4** 7n	**1,2,3**	**1,2,3,4,7n**
P (C1-105) (CDV-P)	**1,2,3,4** 7n	**1,2,3**	**1,2,3,4,7n**
P (16AF10) (16BD3)	**1,2,3,4**	**1,2,3**	**1,2,3,4**
M (C1-144)	**2,3,4,6,8n** [6,8n]	**6,8n**	**3†,4†,8n**
H (C1-15) (16DE6) (16CD11)	**2,3,5** [2†]	**2†**	**2†,3†** 5
F (19BG4)	**2,3,5** [2†]	**2†**	**2†,3†** 5

Bold print denotes commonly observed features.
Normal print denotes less-commonly seen features.
† weakly staining.
[ ] brackets denote a syncytia type.

staining was localized in the central area of large 39°C syncytia with the peripheral areas weakly stained by the fine homogeneous type of staining.

3.4.2.2 Polyclonal Nucleocapsid Antiserum

A very similar pattern of IF staining was obtained with the polyclonal NC antiserum (MeNC-1). Syncytia at 32°C stained with an overall fine homogeneous appearance in addition to the dots and globules, flecks, fibrous material, and fibrous inclusions (Table 5, Fig. 17a). The nuclei of syncytia at both temperatures were observed to have a distinctive diffuse staining like that seen with the anti-M monoclonal antibody (C1-144; Fig. 22a) and occasionally 32°C syncytial nuclei had fluorescent dots (Fig. 17a). The staining seen at 39°C was quite similar to the 32°C staining, although not as heavily flecked. Large 39°C syncytia were characterized by having most of the cytoplasmic staining in the central area of the syncytia thus leaving the edges of the syncytia weakly to moderately stained with the fine homogeneous type of staining (Fig. 17b). Bright nuclear dots were commonly seen at 42 h pi and 39°C but less commonly seen at 27 h pi at 39°C.

3.4.2.3 NP Protein

The 8 h pi sample at 39°C did not stain with either the NP or P (C1-105) monoclonal antibodies. Single cells and early syncytia at 18 h pi (39°C) did show positive fluorescence for the NP and P proteins in the form of cytoplasmic flecks, bright dots and globules.

The NP protein monoclonal antibody (16AC5) intensely stained both the 32°C and 39°C syncytia. The 32°C syncytial cytoplasm contained bright dots and globules, flecks, fibrous material and fibrous inclusions at 51 h pi (Fig. 18a). As well, bright nuclear dots were sometimes seen (Fig. 18a). Comparably sized 39°C syncytia at 27 h pi contained more bright dots and globules and less flecking and fibrous material as compared to 32°C syncytia (Fig. 18b). As time progressed at 39°C, many nuclei had fluorescent dots and syncytia were substantially less flecked and fibrous compared to 32°C infection (Fig. 18c).

Figure 18d depicts an extreme example of the type of syncytium lacking flecks and fibrous material but having some bright dots and globules and distinct nuclear dotting. A comparison of 3+ CPE infected cultures at 32°C and 39°C showed that many more nuclei at 39°C had fluorescent dots than 32°C nuclei. Large 39°C syncytia are also characterized by nonstaining peripheries as the IF staining was localized in the centre of the syncytia.

3.4.2.4 P Protein

Four different clones directed against the P protein were used for IF staining, with slightly different results. The clone designated C1-105 stained the 32°C and 39°C infected cells identically to the described staining of the NP protein (Table 5; Fig. 19a, 19b). The monoclonal antibody directed against the P protein of CDV strongly stained the nuclear dots but only weakly to moderately stained the cytoplasmic material at both temperatures (Fig. 20a, 20b). The other two clones designated 16AF10 and 16BD3 strongly stained the cytoplasmic P protein but did not stain any nuclear material (Table 5; Fig. 21a, 21b). Despite these few differences, all four monoclonal antibodies demonstrated the syncytia at 32°C to be more intensely flecked and fibrous compared to similar sized syncytia at 39°C. Like the NP monoclonal antibody, the P monoclonal antibodies did not stain outer edges of 39°C syncytia (Fig. 20b).

3.4.2.5 M Protein

Staining with the anti-M monoclonal antibody (C1-144) illustrated very different staining patterns at the two temperatures (Table 5). Syncytia at 32°C and 51 h pi were observed to have the following types of cytoplasmic staining: fine homogeneous, flecks, fibrous and fibrous inclusions. As well, the nuclei were seen to be fluorescing with the diffuse type of staining (Fig. 22a). In contrast, 39°C syncytia at 27 h pi had fine homogeneous cytoplasmic staining and very prominent diffuse nuclear (type 8n) staining but lacked the flecks and fibrous material (Fig. 22b). Occasionally this type of syncytia was seen at 32°C. The 39°C syncytia at 42 h pi were characterized by diffuse nuclear staining, pale perinuclear inclusions and pale fibrous material and fibrous inclusions (Fig.

22c). The nuclei in figure 22c are strongly fluoresing and some appear to have tiny dots.

3.4.2.6 H and F Proteins

The three H protein monoclonal antibodies and the F monoclonal antibody produced very similar staining results of the Halle UP-1 infected cells (Table 5). The syncytia at 32°C had intense flecking, fibrous material and fibrous clusters (Fig. 23a, 24a). In contrast, the 39°C syncytia at 27 h pi were weakly stained with pale flecking (Fig. 23b, 24b). Forty-two h pi syncytia stained with approximately one-third the intensity of 32°C syncytia and were characterized by weak flecking and fibrous material and the occasional fibrous cluster. Sometimes the 32°C cultures were observed to contain the weakly staining 39°C type of syncytia.

3.4.3 Electron Microscopic Observations

Nucleocapsid material was present in the cytoplasm of Halle UP-1 infected cells at 32°C (4+ CPE) and budding and extracellular virus were a prominent feature of these cells. The appearance of the extracellular virus and active plasma membranes was like that seen at 72 h pi in RP-infected cells at 32°C (Fig. 14b). Halle UP-1(32) occasionally contained simple NB.

A few simple NB were found in the preparation of uninfected control Vero cells at 39°C and 3 d pi. Simple NB were more numerous in Halle UP-1(39) (4+ CPE) and as many as three bodies could be seen in individual nuclei (Fig. 25c). Nucleocapsid tubules were not seen in Halle UP-1(39) nuclei but early signs of nucleolar destruction were observed (Fig. 25c). Although NC material was seen in the cytoplasm of these 39°C infected cells, no budding or extracellar virus was detected. Cytoplasmic NC was primarily found in syncytial cells and ranged in amount from small, tightly packed inclusions (Fig. 25a) to entire cytoplasms full of "fuzzy" NC (Fig. 25b).

3.5 Halle Undiluted Passage-2 Study

Halle UP-1 infectious material prepared at 39°C was used in undiluted form to infect Vero cell monolayers on coverslips and in Blake bottles at an moi of approximately 10^{-3} to 10^{-4} pfu/cell. Two different passage levels of cells (p. 144 and p. 170) were used and infected cells were incubated at 32°C and 39°C. Uninfected control cells were maintained at 32°C and 39°C. Photographs were taken of the progression of the CPE and HAD results. At various times post infection, coverslips were fixed and used for IF assays with the following monoclonal antibodies: NP (16AC5), P (16AF10), M (C1-144), H (C1-15) and F (19BG4).

Infected cells in Blake bottles at 39°C were trypsinized and passaged 5 d pi and approximately at nine day intervals thereafter. Resuspended cells were seeded to Blake bottles or large flasks and Leighton tubes containing coverslips which were subsequently incubated at 32°C and 39°C. The cells infected at passage 144 were passaged four times and the infected passage 170 cells were passaged nine times. Immunofluorescence assays were performed on the prepared coverslips at each passage and temperature. An electron microscope sample was prepared of the third passage at 39°C (11 d pp) of the cells originally infected at passage 144.

3.5.1 Development of Cythopathic Effect and Hemadsorption

The UP-2 infection at 39°C was characterized by limited syncytia development involving less than 50% of the monolayer. This infection displayed the "healing syncytial hole" phenomenon and levels of infectious virus which were at the limit of detection of the plaque assay system used.

Syncytia were first seen 1 to 2 d pi in the UP-2(39) culture (Fig. 26a). The syncytia did not enlarge indefinitely but detached from the substrate and left a hole or plaque in the monolayer. These syncytial holes, seen as early at 3 d pi, are then filled by the inward growth and multiplication of the surrounding cell monolayer (Fig. 26c, 26e). This process was termed "healing". Some holes are completely filled by 5 d pi and at 7 d pi the appearance is that of a complete monolayer plus the occasional syncytium and plaque. The development of the syncytia, the syncytial detachment and the healing process occurs simultaneously in the UP-2

culture as a few new syncytia are always forming. Shift-down of UP-2 infected cells to 32°C resulted in total destruction of the monolayer in seven to ten days and production of high levels of infectious virus. It was observed that the healed syncytia areas do not reactivate to active syncytia when shifted down to 32°C.

The syncytia produced at 39°C were HAD positive (Fig. 26b) whereas the healed syncytia were HAD negative (Fig. 26f). The holes in the process of filling were either HAD negative or weakly positive (Fig. 26d). The UP-2 cultures grown at 32°C were strongly HAD positive.

An unusual amount of CPE was seen in the third passage (p. 147) of Halle UP-2 cells originally infected at passage 144. The passage 147 cells incubated at 39°C developed syncytia involving 75% of the monolayer at 2 d pp. By 5 d pp only 50% of the monolayer in the form of spindly cells remained attached to the substrate and extracellular virus was produced (1.7 X 10^2 pfu/mL). The monolayer did not recover in the following days and a few active syncytia were seen at 11 d pp at which time one flask was prepared for electron microscopy and another flask was passaged. These passaged cells did not grow well at 32°C or 39°C.

The cells infected at passage 170 were further passaged nine times and gradual changes were noted in the CPE development. The fifth passage demonstrated plaque healing at 39°C and extensive CPE at 32°C but by the seventh passage, few of the plaques were healing at 39°C and the CPE at 32°C did not progress to 100% involvement. Limited CPE (1+) was observed 9 d pp of the eighth passage at 32°C. Syncytial holes were not healing at the ninth passage at 39°C although some active syncytia were seen.

3.5.2 Immunofluorescent Staining

Studies on UP-2 cultures or passages thereafter showed distinct differences between the IF staining pattern obtained with syncytia produced at 32°C and syncytia and healing syncytial holes produced at 39°C. Table 6 summarizes the staining patterns observed with five monoclonal antibodies against the UP-2 infection and passage thereafter.

Table 6

Halle UP-2 immunofluorescent staining

Antiserum	32°C	39°C Syncytia	39°C Holes
NP (16AC5)	**1,2,3,4**	**1*,6**	**N**
	7n, [1*,6]	7n, [2,3,4]	7n
P (16AF10)	**1,2,3,4**	**1*,6**	**N**
	[1*,6]	[2,3,4]	
M (C1-144)	**2,3,4,6,8n**	**6,8n**	**N**
	7n, [4†,6,8n],[6,8n]	3†,4†,7n, [8n†]	
H (C1-15)	**2,3,5**	**2†,3†**	**N**
	[2†]	[2,3,5]	
F (19BG4)	**2,3,5**	**2†,3†**	**N**
	[2†], [N]	[N]	

Bold print denotes commonly observed features.
Normal print denotes less-commonly observed features.
* few inclusions.
† weakly staining.
N nonstaining.
[ ] brackets denote a syncytia type.

3.5.2.1 NP Protein

The NP specific monoclonal antibody strongly stained the 32°C maintained infected cells both cytoplasmically and, occasionally, intranuclearly. The cytoplasmic staining was in the form of bright dots and globules, flecks, fibrous material and fibrous inclusion bodies. Bright dots were seen in some nuclei. In contrast, the staining intensity was noticeably reduced in the infected 39°C culture. The 39°C syncytia were weakly to moderately stained and generally lacked the flecked and fibrous appearance seen at 32°C. At 39°C, the syncytial cytoplasm showed a fine homogeneous type of staining with fewer bright dots and globules (Fig. 28a). As well, the fluorescence was concentrated in the centre of large syncytia with the syncytial edges nonstaining. Intranuclear dots were sometimes seen in active syncytia at 39°C. Occasionally a syncytium was observed at 39°C which had the staining characteristics of the "typical" 32°C syncytium. It was also possible to find a few syncytia in the 32°C cultures which displayed the typical staining associated with the 39°C infection (Fig. 28b). The healing syncytial plaques were not stained by the NP monoclonal antibody. An exception was found in the first passage of a UP-2 culture at 39°C in which a few individual cells on the edge of one healing hole were found to contain distinct intranuclear dots but not cytoplasmic staining (Fig 27c).

3.5.2.2 P Protein

The staining obtained with the P component specific monoclonal antibody (16AF10) was similar in description and intensity to that obtained for the NP protein with the exception that this clone did not stain intranuclear inclusions (Table 6). Syncytia in the 32°C maintained cultures were primarily the intensely stained type and 39°C syncytia were characterized by pale to moderate intensity fine homogeneous cytoplasmic staining with some dots and/or globules (Fig. 27). Sometimes the typical 39°C type of syncytia was observed at 32°C and typical 32°C syncytia could be found at 39°C. The healing syncytial holes were not stained by this P monoclonal antibody (Fig 28a).

3.5.2.3 M Protein

Syncytia produced at 32°C showed bright cytoplasmic staining characterized by flecks, fibrous material, fibrous inclusions and an overall fine homogeneous staining. Nuclei displayed a diffuse staining around the nucleoli not observed in non-fused neighbouring cells or uninfected controls (Fig. 30d). Occasionally, weakly stained syncytia like the type seen at 39°C were found in 32°C cultures. Syncytia produced at 39°C stained with a much duller intensity compared to the 32°C infection. The cytoplasmic area of the 39°C syncytium was non-stained to weakly stained with occasional fibrous-type material or pale perinuclear inclusions. The nuclei within the syncytia showed the diffuse staining previously described. In the passage 147 UP-2 cultures, the M monoclonal antibody (C1-144) stained distinct nuclear inclusions like those stained by the NP and P monoclonals. The nuclear dots were more predominant at 39°C (Fig. 30a, 30b) but were also seen at 32°C (Fig. 30c). Two other passages of the UP-2 cultures also showed nuclear dots although not as clearly as the third passage material. Healing holes at 39°C were not stained by the M monoclonal antibody (Fig. 29b).

3.5.2.4 H and F Proteins

The monoclonal antibodies against the H and F components stained the UP-2 cultures in a similar manner. Both antibodies strongly stained syncytia at 32°C and gave the appearance of flecks, fibrous material and fibrous clusters whereas the 39°C syncytia were usually weakly stained with pale flecks and fibers. A few weakly flecked syncytia were found in the 32°C cultures and the occasional brightly stained (H protein) area was seen at 39°C. Some syncytia at both temperatures appeared not to be stained with the F monoclonal. Nuclear staining with the H and F antibodies was not seen at either temperature and healing holes were not stained by either antibody.

3.5.3 Electron Microscopic Observations

The cells and scattered syncytia which remained attached to the plastic flask 11 d pp of the passage 147 UP-2(39) were observed by electron microscopy. Nucleolar destruction was

commonly seen (Fig. 31b) and margination of chromatin was infrequently observed. No cytoplasmic NC material or extracellular virus was observed. An estimated 5-10% of the cells contained simple NB and the complex forms of NB were less frequently seen. Figure 31a illustrates a complex NB of the beaded variety and figure 31b illustrates another complex NB which appears to contain some type of tubules and may be a viral NB.

4. Discussion

The aim of this project was to study MV-H persistently and lytically-infected Vero cells using the techniques of immunofluorescence and electron microscopy. Immunofluorescent staining of MV-H infected cells has demonstrated the distinct and reproducible staining patterns of the viral NP, P, M, H and F proteins. The staining patterns obtained for the NP and P proteins were very similiar to each other. As well, the stained appearance of the H protein was similar to the appearance of the F protein. The M protein staining was unique from the other four proteins. The observations made during this study support published information as well as offering new information. Clear differences were seen in the IF staining of 32°C syncytia and 39°C syncytia in Halle UP-1 and UP-2 cells. The healing syncytium phenomenon reported here has been previously described in the literature but only as seen in cell-associated SSPE cell lines (Burnstein et al., 1974; Makino et al, 1977; Norrby et al., 1985). A detailed description of this limited syncytial growth and subsequent healing of the plaque has not been previously reported.

The gene order from the 3' end of the MV genome is NP, P, M, F, H and L (Richardson et al., 1985). The order of appearance of the five viral proteins in the RP growth study at 32°C, as determined by IF assay, correlated closely with the known MV gene sequence. For example, NP protein was the first protein detected (6 h pi) and it is located at the 3' end of the virion RNA. The next protein detected was the P protein at 10 h pi followed by the M protein at 18 h pi. The glycoproteins H and F were detected at 20 and 21 h pi respectively and the L protein was not assayed by IF due to a lack of L protein specific monoclonal antibody.

The early appearance of the NP protein in RP-infected cells agreed with the IF detection of the N protein in Sendai virus-infected cells at 7 to 8 h pi (Dubois-Dalcq et al., 1984). In the present study, positive staining of MV-H-infected cells was obtained with an NP monoclonal antibody prepared against the MV-L strain thus supporting the observation of antigenic stability of the NP protein (Sheshberadaran et al., 1983). The intranuclear fluorescing dots containing NP protein observed in this study, have been reported in the

literature and are usually associated with persistence. Giraudon et al. (1984) reported less than 5% of MV-H lytically infected cells contained intranuclear NP dots whereas these dots were contained in up to 50% of MV-H persistently infected cells. In the present study, nuclear dots which contained NP antigen were observed in both Halle UP-1 and UP-2 cells but were not detected in RP-infected cells at 32°C. The reason for this difference is unclear and may be related to the inoculum virus. For example, the UP-1 and UP-2 cultures were infected with inocula containing both DI particles and standard virus whereas the RP experiment used inoculum which was prepared at 32°C and probably contained few DI particles compared to standard virus.

This and other studies have noted that the P antigen in MV-infected cells is localized in a pattern indistinguishable from the NP antigen (Johnson et al., 1982; Giraudon et al., 1984). This observation suggests a close structural relationship between P and NP proteins (Johnson et al., 1982). The fluorescing nuclear dots containing P protein which were observed in the Halle UP-1 cultures have not previously been reported in the literature. The present observation that the polymerase P protein was localized intranuclearly and intracytoplasmically with two anti-P monoclonal preparations (C1-105 and CDV X/32) but only intracytoplasmically with two other clones (16AF10 and 16BD3) was unexpected. This could indicate that some of the P protein epitopes were altered when the component moved into the nucleus and only certain monoclonal antibodies could recognize the P protein in both sites. This same phenomenon has been seen with the NP protein in CDV-infected cells (Örvell, Sheshberadaran and Norrby, 1985) and the C protein in MV-infected cells (Bellini et al., 1985). The specificity of the C1-105 and CDV-P X/32 clones should be rechecked to verify the intranuclear localization of P protein.

Dubois-Dalcq et al. (1984) reported that it was unclear whether the M protein was associated with the cytoplasmic NC. Data from the present study suggests that the M protein is associated with the cytoplasmic NC material as shown by the staining of perinuclear inclusions (Fig. 11e, 22c) typically stained by NP and P monoclonal antibodies. Johnson et al. (1982) also found that the M protein was commonly located in perinuclear inclusions or as

diffuse granular cytoplasmic material. The fact that NP and P proteins are components of the NC and that M protein contaminates purified NC lend further support to this association between NC material and M protein. M protein sometimes appeared to be membrane associated like the H and F proteins (Dubois-Dalcq et al., 1984). Membrane staining with the M monoclonal antibody (C1-144) was characterized by the fibrous cluster type of staining. The M protein containing fibrous clusters were a prominent feature of MV-Lec-infected cells and were seen in Halle-infected cells. The conclusion that the M protein of MV is the most antigenically variable component (Sheshberadaran et al., 1983) was supported by the present observation that Halle-infected cells could be stained with Halle-specific M monoclonal antibody but were not stained with Lec-specific M monoclonal antibodies (19CG6 and 19DC5).

The observation of diffuse nuclear staining in Halle and Lec-infected cells with the anti-M monoclonal antibody (C1-144) has not been reported in the literature although it has occasionally been seen in MV and CDV-infected cells (E. Norrby, unpublished observations). The immunofluorescent nuclear localization of the M protein in Sendai virus-infected cells with a polyclonal Sendai M protein antiserum has been reported (Yoshida et al., 1976). Staining appeared as diffuse nuclear staining with slightly brighter patches within the nucleus (Yoshida et al., 1976). The possibility that this M protein clone (C1-144) might stain nuclei and cytoplasm due to a cross-reaction with a cellular stress protein was tested. Stress proteins accumulate in avian and mammalian cells in response to a variety of stresses ie., heat shock, heavy metals, sulfhydryl reagents, amino acid analogs and tissue explantation (Collins and Hightower, 1982). Some stress proteins are glucose-regulated proteins which are synthesized at increased rates in mutant cells types, during paramyxovirus infection and during glucose starvation and exposure to inhibitors of glycosylation, ie., 2-deoxyglucose and tunicamycin (Collins and Hightower, 1982). Infection by a number of paramyxoviruses has stimulated the synthesis of cellular stress proteins (Peluso, Lamb and Choppin, 1978; Collins and Hightower, 1982; Sheshberadaran and Norrby, 1984). The function of stress proteins and the mechanisms responsible for their induction during viral infections are not known (Collins and Hightower,

1982). In the present study cells were stressed by infecting them with mumps virus, a paramyxovirus. Since measles and mumps viruses are not considered to be antigenically related, any positive staining of mumps virus-infected cells with the C1-144 clone would appear to be a nonspecific cross reaction with a cellular stress protein. The C1-144 anti-M clone did not appear to cross-react with a cellular stress protein as mumps virus-infected cells did not stain intranuclearly or cytoplasmically (Fig. 15c). The observation of distinct intranuclear dotted fluorescence in some of the UP-2 passaged cells also seemed to indicate that the diffuse nuclear staining was not the result of a non-specific cross-reaction with a cellular stress protein. The significance of this rarely-seen nuclear dotting in UP-2 passaged cells, which was most clearly seen in passage 147 cells, is unknown. It was clear, however, that the viral infection was stimulated in these passage 147 cells as the 39°C culture developed an unusual amount of CPE (3+) compared to other passages at 39°C. It would be desirable to obtain additional M protein specific monoclonal antibodies, in particular those which Dr. Norrby reported to give similar diffuse nuclear staining, to see if they would also stain the Halle-infected cells as reported in this study.

The intranuclear localization of the NP, P and M proteins by immunofluorescence was supported by studies of intranuclear MV-specified polypeptides (Wechsler and Fields, 1978; Miller, 1980). These authors showed that purified nuclei of MV-infected cells contained the L, P, NP, actin and M viral polypeptides by polyacrylamide gel electrophoresis of radio-labeled cell extracts. The role of the viral proteins in the cell nucleus is unclear. The nuclear proteins are not believed to be involved in virus assembly and may represent excess protein due to the decreased or absent viral budding. This hypothesis is supported by the present observations of NP protein staining in the UP-1(39) and UP-2(39) syncytia. Syncytia in the UP-1(39) cultures contained large amounts of NP protein in the cytoplasm and were observed to commonly contain NP protein in the nuclei at 42 h pi. In contrast, syncytia in the UP-2(39) culture appeared to contain less cytoplasmic NP protein and only occassionally were dotted nuclei seen. Nuclear functions are not necessary for the replication of Paramyxoviridae (Dubois-Dalcq et al.,1984; Follett et al., 1976). Measles virus-infected enucleate cells

produced 100-fold less infectious virus and 20% of the polypeptides compared to nucleated cells (Follett et al., 1976). It is thought that these effects may result from damage to the cellular organelles during enucleation (Dubois-Dalcq et al., 1984).

The fleck-type staining obtained with the H protein monoclonal antibodies and the F protein monoclonal antibody corresponded to the trabeculated (small rod) staining of H and F proteins in MV-infected cells (Norrby et al., 1982). In the present study, flecks appeared to aggregate into fibrous material which subsequently coalesced into fibrous clusters. The fibrous clusters were differentiated from fibrous inclusions (NP, P and M proteins) as the clusters appeared to be at the cell surface whereas the fibrous inclusions were located cytoplasmically. Similar clusters of H protein were also observed in the plasma membrane of unfixed Halle-infected cells (Giraudon et al., 1984). Rentier et al. (1978) reported that HAD sites moved towards the center of the syncytia which explains why adsorbed erythrocytes were often grouped in the center of syncytia (Fig. 26b).

The IF staining results on Halle UP-1 and UP-2 cells illustrated distinct differences in the staining patterns and accumulations of the viral proteins at 32°C and 39°C. Decreased staining of the M, H and F proteins was clearly seen in the UP-1(39) culture compared to the UP-1(32) culture. These observations were in agreement with the decreased or negative IF staining of M, H and F proteins in moderately productive and nonproductive SSPE virus-infected cells (Johnson et al., 1982). A decreased amount of fluorescence obtained with a monoclonal antibody is usually taken to mean a decreased amount of the specific protein present in the infected cells. The Halle UP-1(39) culture appeared in this present study to have decreased levels of the M, H and F proteins as compared to the lytic infection at 32°C. Decreased amounts of these viral proteins could account for the 10,000-fold reduction in infectious virus as compared to the 32°C infection. Morphogenesis of infectious virions is dependent upon the insertion of both H and F glycoproteins into the plasma membrane and the functioning of the M protein to align NC material beneath the modified areas of the cell membrane. Reduced amounts of these proteins could certainly reduce the number of budding virus.

Immunofluorescent staining of Halle UP-2 cultures and passages thereof, demonstrated the decreased staining of all five proteins (NP, P, M, H and F) at 39°C compared to the lytic infection at 32°C. Some syncytia were not stained with the F monoclonal antibody despite the obvious biological activity of the F protein. Either the number of F proteins had fallen below the sensitivity of the IF assay or the epitope had been altered such that the clone no longer recognized the protein. The Halle UP-2(39) cultures appeared to contain decreased levels of the NP, P, M, H and F proteins. Reduced amounts of these proteins would serve to decrease the level of infectious virus produced. The UP-2(39) staining pattern obtained with the NP and P monoclonal antibodies was very different from that obtained in the UP-1(39) cultures. The syncytia at 39°C (UP-2) were characterized by few bright dots and globules with the majority of the staining in the form of diffuse cytoplasmic fluorescence. This lack of brightly stained globules may be explained by a lack of cytoplasmic NC inclusions which was supported by the electron microscopic observations on the passage 147 UP-2(39) cells. Cytoplasmic NC was not detected in these cells. This could be explained by the failure of the nucleocapsid proteins to encapsidate viral RNA or a lack of viral RNA for encapsidation.

The similarities between the MV-H cytopathic growth patterns observed in this study and that reported by Burnstein et al. (1974) were striking. Burnstein et al. (1974) studied primary SSPE brain cell cultures and the persistently-infected cell line IP-3 which was developed from the co-cultivation of SSPE brain cells with monkey kidney cells (BSC-1). IP-3 cultures and Halle UP-2 cultures maintained at 39°C did not produce detectable infectious virus and displayed a cycling pattern of CPE characterized by the formation of syncytia, necrosis of syncytia leading to plaques and the subsequent healing of the plaque. Measles antigen, as detected by IF assay, was present only in syncytia. The primary brain cell cultures also displayed the healing syncytium phenomenon but no further lesions beginning a second cycle.

The Halle UP-2 immunofluorescence studies have shown that the cells which fill in the hole in the monolayer did not stain for the MV NP, P, M, H and F components. This was

supported by the observation that large syncytia at 39°C had the majority of IF staining localized in the center of the syncytia while the edges of the syncytia and neighbouring non-fused cells were nonstained by component specific monoclonal antibodies. This pattern of staining may be explained by the diffusion of the F glycoprotein through the fluid lipid bilayers of the plasma membrane to neighbouring cells (E. Norrby, personal communication). This movement would cause membrane fusion in the absence of other viral proteins. Although the cell membranes on the periphery of the syncytia have fused, viral proteins were not detectable indicating that the cells which fill in the plaques were not infected. This conclusion was supported by the observation that healed syncytial holes, when shifted down to 32°C, did not reactivate. This persistent infection was believed to be maintained by the continual production of low levels of infectious virus; the levels being below the limits of detection (<10 pfu/mL) of the plaque assay system.

The Halle UP-2 passage 147 cells at 39°C which developed an unusual amount of CPE involving about 75% of the monolayer, were indicative of the "crisis" stage that newly established persistent infections often display. A possible explanation may be related to the cyclical variation in the amount of standard virus and DI particles as seen in the Halle ring plaque phenomenon. The cyclical variation of DI particles and standard virus may occur slowly over a number of passages, ie with the number of standard viruses increasing and the number of DI particles decreasing, until the ratio of standard virus to DI particles is maximal with the resulting extensive CPE. Possibly related to this cycle was the seldom seen reactivation at 39°C of healed and healing holes in the second passage of a Halle UP-2 culture. Seven of eight healing and healed holes were seen to become active syncytia at 8 d pp at 39°C. This was followed by necrosis of the syncytia, formation of plaques and healing at 14 d pp.

Electron microscopic observation of MV-H infected cells demostrated fuzzy NC material in the cytoplasm of RP-infected cells at 32°C (72 and 96 h pi), UP-1(32) and UP-1(39) cultures. Cytoplasmic fuzzy NC in Halle-infected cells have been previously reported (Hamilton, Barbosa and Dubois, 1973; Minagawa et al., 1976; Wild and Dugre, 1978). The arrangement of the NC tubules observed in the present study varied from small,

tightly packed inclusions (Fig. 25a) to entire cytoplasms full of fuzzy NC (Fig. 25b). The small, tightly packed inclusions may correspond to the brightly fluorescing dots and globules seen with the NP and P protein IF staining. Budding virus seen in RP-infected cells at 32°C and the UP-1(32) culture correlated with the production of high titers of virus (10^6 - 10^7 pfu/mL). In contrast, budding virus was not observed in the Halle UP-1(39) culture which produced low levels of virus (10^3 pfu/mL) and contained cytoplasmic NC material.

Simple NB, with a fibrillar appearance, were seen in RP-infected cells at 32°C (96 h pi), uninfected 39°C control cells, Halle UP-1(32), UP-1(39) and UP-2(39;p. 147). The observation of a small number of simple NB in the uninfected Vero cells is in agreement with the comment of Bouteille et al. (1974) that many, if not all, types of normal cells contain simple NB. These NB are more numerous in stimulated cells (i.e. viral stimulated cells) as was seen in the Halle UP-1(39) cells which contained as many as three simple NB per nuclei (Fig. 25c). It was noticed by Fournier et al. (1980) that the percentage of nuclei containing at least one simple NB increased with time post infection. Oyanagi et al. (1970) reported that the nuclear inclusions (containing NC) corresponded to the eosinophilic and fluorescent nuclear inclusions in cell cultures derived from SSPE brain biopsies. The simple and complex NB observed in the present study may correspond to the fluorescent nuclear dots obtained with the polyclonal antisera and NP and P monoclonal antibodies. The intranuclear organization and relative sizes of the fluorescing dots and NB appeared to be similar. Probably the viral proteins were synthesized in the cytoplasm and then diffused across the nuclear envelope to accumulate inside the nucleus. Whether the NP and P proteins were attracted to a pre-formed NB or their close structural relationship caused the two proteins to aggregate thus forming a simple NB is unknown. The latter explanation seemed more probable as the number and complexity of NB in MV-infected cells increased with time post infection. The diffuse nuclear localization of M protein observed in this study may be explained by a lack of attraction between the M protein and the aggregates of NP and P proteins. Perhaps the sites on the nucleocapsid proteins which normally bind to the M protein were masked or in a different conformation in the nuclei. This situation was observed to change in the passage 147

Halle UP-2(39) cells in which M protein was localized in distinct fluorescent dots like the NP and P proteins. This seemed to indicate that the M protein was now attracted to the NP and/or P proteins perhaps due to a conformational change in the M, NP or P protein or unmasking of a specific binding site. Electron micrographs of these cells demonstrated complex NB, as well as simple NB, possibly indicating sites of early NC assembly. Perhaps this combination of viral RNA, NP and P proteins is necessary for the attraction of M protein.

Intranuclear NC tubules were not clearly detected in this study although they have been observed in MV-H infected cells (Hamilton et al., 1973; Minagawa et al., 1976; Wild and Dugre, 1978). Hamilton et al. (1973) studing Halle-infected Vero cells, observed smooth NC either dispersed within the nucleus or forming small aggregates within nuclear bodies (viral NB). The MV-H persistently infected African green monkey kidney cell line (BGM/Halle) was observed at the eleventh passage to contain large nuclear aggregates of smooth filaments (15-18 nm) and cytoplasmic granular filaments (18-25 nm) (Wild and Dugre, 1978). Most BGM/Halle cells contained the NC material in either the cytoplasm or nucleus, and few cells had NC in both locations. Minagawa et al. (1976) reported that NC tubules were seldom observed in the nuclei of MV-H persistently infected human lymphoid cells but were commonly seen in Mantooth (SSPE isolate) persistently infected lymphoid cells. These differences in the occurence of intranuclear NC material are probably dependent upon the type of host cell used, the temperature of cultivation and the passage history of the virus.

Oyanagi et al. (1970) hypothesized that NB could represent early foci in the formation of NC. This is supported by the fact that viral NC material has been associated with NB. Viral NB, characterized by NC tubules surrounded by a fibrillar envelope, have been observed in cell lines from SSPE brain biopsies (Oyanagi et al., 1970) and MV-infected cells (Hamilton et al., 1973; Fournier et al., 1981). A complex NB (Fig. 31b) was seen in the present study in the passage 147 Halle UP-2(39) cells which appeared to contain tubular structures having a diameter of approximately 13.5 nm. This estimate was on the low end of the reported diameter of 15-18 nm for smooth NC filaments (Wild and Dugre, 1978). This

complex NB appeared to be a developing viral NB. Fournier et al. (1981) postulated that NB may act as specific target organelles for viral infection and/or the possible sites for the assembly of nucleocapsids, proteins and MV RNA.

The Halle strain was originally isolated from a lymph node of an SSPE patient. Lymph node cells were co-cultivated with HeLa cells at 37°C with resulting syncytia within 2 to 3 days and infectious virus produced. HeLa monolayers infected with this media showed extensive CPE (2-3 d pi) and high levels of infectious virus (10^8 pfu/mL). The Halle isolate was a very cytopathic strain. In the present study, undiluted passage of MV-H at 37°C and 39°C produced rapid changes in the characteristics of the virus, even after one passage (ie. UP-1(39)). After two undiluted passages at 39°C, the Halle infected cells resembled certain nonproductive SSPE co-cultures and the primary SSPE brain cell culture reported by Burnstein et al. (1974) which were characterized by the healing syncytial plaques. The Halle SSPE isolate is known to produce DI particles in cell culture (Tsang et al., 1981; McKimm-Breschkin et al., 1982). Incubation of the virus at 37°C induced the ring plaque phenomena which has been shown to be asscciated with the temperature-dependent production of interfering particles (Tsang et al., 1981). Undiluted passage at 39°C caused the production of DI particles which initiated the persistent infection of Vero cells by the MV Halle strain. It was unlikely that large numbers of ts mutants gave rise to these persistently-infected cells since the titer at 32°C and 37°C of UP-1 virus stock was similar (Fig. 3). The location of the block in production of the viral proteins at 39°C is unknown, though both transcriptional and translational events may be involved.

5. Conclusion

The immunofluorescent detection of the P, M and NP protein, in the nuclei of MV-infected cells is new information obtained in this study. The role of these nuclear proteins is unclear but is not believed to be involved in virus assembly. The fact that NP protein intranuclear localization is more common in cells harbouring a defective MV infection may indicate a relationship between low virus production and intranuclear accumulation of viral proteins. A possible explanation could be the diffusion of NP, P and M proteins into the nucleus from a cytoplasm containing excess viral proteins due to the lack of or decreased viral budding. The H and F proteins probably would not be free to diffuse into the nucleus because these proteins are attached to the membranes of the rough endoplasmic reticulum, golgi apparatus and plasma membrane. Thus the nuclei may simply be passive sites of accumulation of excess viral proteins and viral RNA, rather than be actively involved in controlling persistence in some way.

This study reports for the first time the antigenic status of the cells which have been observed to grow into the plaques produced by degenerating MV syncytia. The cells which fill the plaque in the monolayer appeared to be noninfected as determined by IF assay and shift down experiments to 32°C.

Undiluted passage of MV-H at 39°C produced noticeable changes in the characteristics of the infection, even after one passage. Two undiluted passages at 39°C produced the phenomenon of healing syncytium. The rapidity of the change was believed due to the production of DI particles. Perhaps the production of DI particles plays a role in the etiology of the disease SSPE. The persistent MV infection of SSPE could possibly be established by the production of DI particles in the CNS tissue. These DI particles could act as natural modulators of the MV infection, thus providing protection for the host. The MV infection of SSPE brain cells is known to be defective as infectious virus has not been isolated directly from brain tissue but requires co-cultivation techniques. If DI particles are involved in the etiology of SSPE, then the possibility exists that there is a continous dynamic interaction between the standard virus and its DI particles. Such a cycling pattern has been observed in

the brains of mice inoculated with a mixture of standard VSV virus and its DI particles (Cave et al., 1985). Cyclic production of DI particles which contain functional genes may produce quantitative differences in the appearance of gene products (Cave et al., 1985). DI particles which do not contain functional genes may also lead to a decrease in the expression of certain genes. These cultures appear to have decreased production of the M, H and F proteins and also the NP and P proteins in the case of the UP-2 culture.

Little is known about MV DI particles. Most of the current knowledge has come from studies on the DI particles of VSV and Sendai virus. Generally, DI particles may be missing any gene/s or segments thereof. A Halle DI particle composed of the 3' end of the parental RNA and lacking the genomic sequences for the M, F and H proteins would explain the apparent decreased production of those proteins in the Halle UP-1(39) cultures. Perhaps a different DI particle which was missing even a larger portion of the genomic RNA, namely the NP, P, M, F and H protein genes, was prominent in the UP-2(39) cells accounting for the apparent decreased synthesis of all five proteins. Whether the Halle DI particles in this study contain fusion DI, 3' terminus or 5' terminus RNA or a combination of these is unknown. An examination of the genetic content of the DI particles in this study was beyond the scope of this project but could in the future be characterized by hybridization, oligonucleotide fingerprint analysis and direct sequencing analysis.

Further experiments could be carried out to determine the location of the block leading to decreased amounts of the various proteins. In vitro translation of mRNAs isolated from UP-1(32) and UP-1(39) infected cells would demonstrate the presence or absence of specific mRNA and their relative amounts at the two temperatures. The presence of similar amounts of a specific mRNA (ie. H protein mRNA) in UP-1(32) and UP-1(39) cultures but the synthesis of low levels of H protein at 39°C compared to 32°C would indicate a translational problem at 39°C. Agarose electrophoresis of these mRNA preparations would show if the sizes or amounts of the specific mRNAs differed in UP-1 cultures at 32°C and 39°C. The absence or decreased production of a specific mRNA at 39°C could indicate a transcriptional blockage or possibly lack of the complementary genomic RNA, either of which

could explain the decreased production of viral proteins at 39°C as compared to 32°C. Another method which could be used to detect specific mRNAs involves the use of radiolabelled MV probes. Probes prepared against MV mRNAs could be used in spot hybridization techniques. Positive hybridization would indicate that specific mRNAs were present in the infected cells.

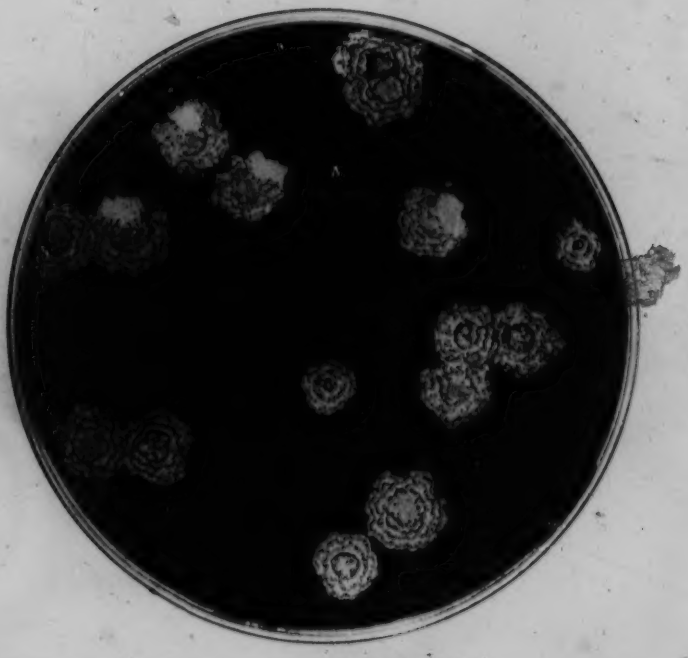
The methodologies used in the present study have inherent strengths and limitations. Both fluorescence microscopy and electron microscopy are visual techniques which allow one to observe directly some effects of virus infection upon cells. Immunofluorescence is a fast, simple technique which can be applied to any virus-cell system if virus-specific antiserum is available. The advent of monoclonal antibodies has certainly increased the utility of this technique. Electron microscopy allows visualization of cellular and viral components and when combined with immune labelling techniques, is a very powerful tool. The major limitation of the techniques used in this study is that they do not provide molecular or biochemical information which was beyond the aim of this study.

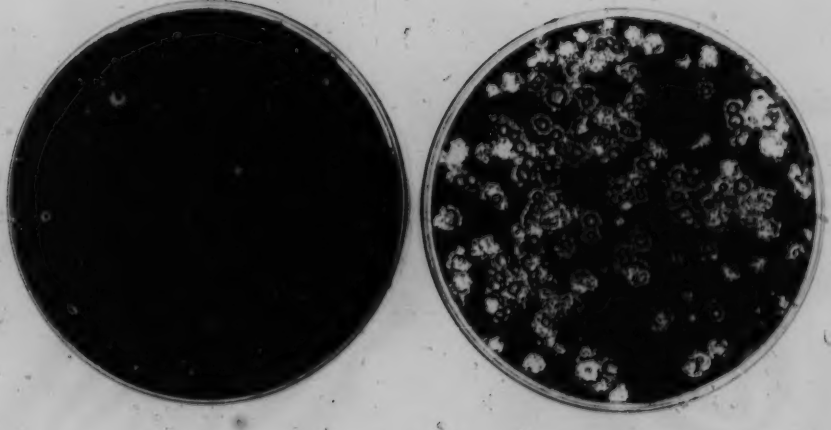
Figure 2a.

A photograph of a fixed and stained plaque assay dish which was inoculated with MV-H and incubated at 37°C. This dish illustrates the "ringed plaques" with alternating rings of living and dead cells. Refer to section 2.5 for the methodology used.

Figure 2b.

A photograph of plaque assay dishes inoculated with MV-H IM prepared at 32°C. Dishes incubated at 37°C displayed ring plaques whereas dishes incubated at 32°C displayed small plaques.





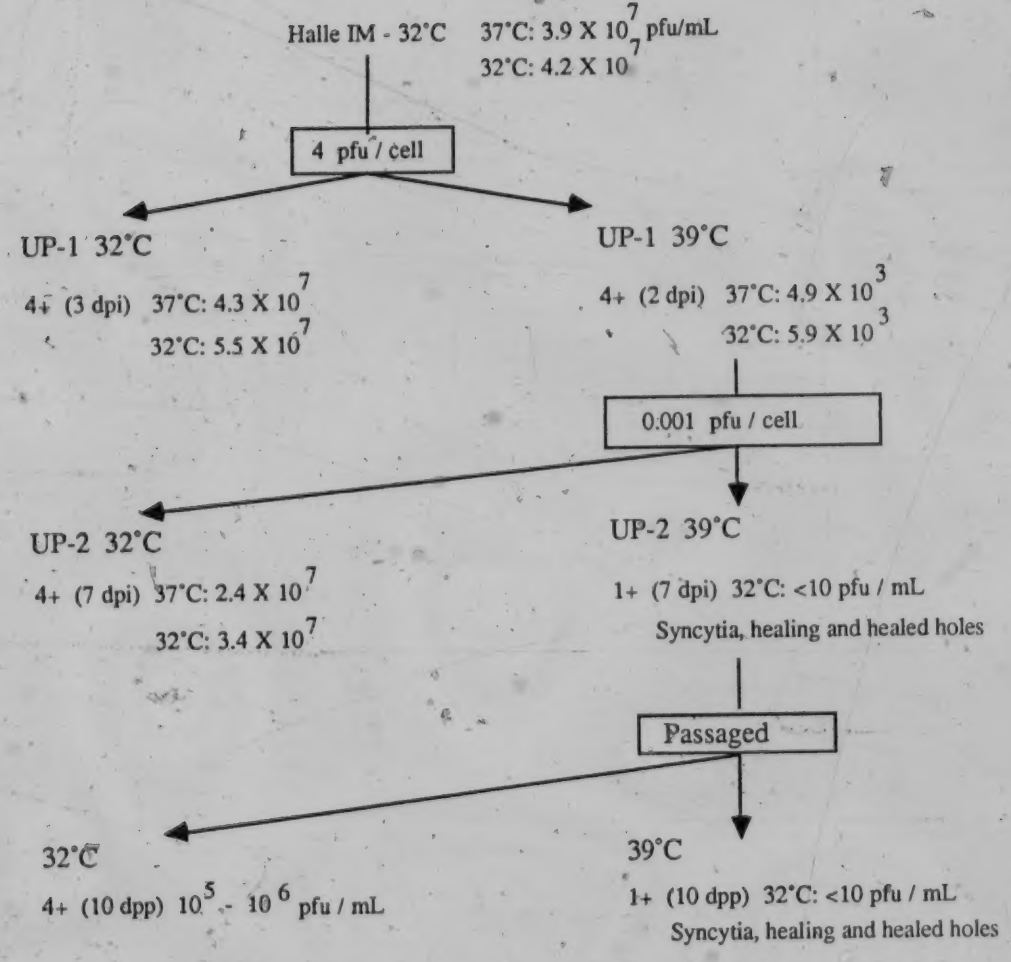

Halle IM - 32°C
37°C: 3.9 X 10^7 pfu/mL
32°C: 4.2 X 10^7
4 pfu / cell
UP-1 32°C
4+ (3 dpi) 37°C: 4.3 X 10^7
32°C: 5.5 X 10^7
UP-1 39°C
4+ (2 dpi) 37°C: 4.9 X 10^3
32°C: 5.9 X 10^3
0.001 pfu / cell
UP-2 32°C
4+ (7 dpi) 37°C: 2.4 X 10^7
32°C: 3.4 X 10^7
UP-2 39°C
1+ (7 dpi) 32°C: <10 pfu / mL
Syncytia, healing and healed holes
Passaged
32°C
4+ (10 dpp) 10^5 - 10^6 pfu / mL
39°C
1+ (10 dpp) 32°C: <10 pfu / mL
Syncytia, healing and healed holes

Figure 3

The undiluted passage of MV-H and the production of persistently - infected cells

Figure 4.

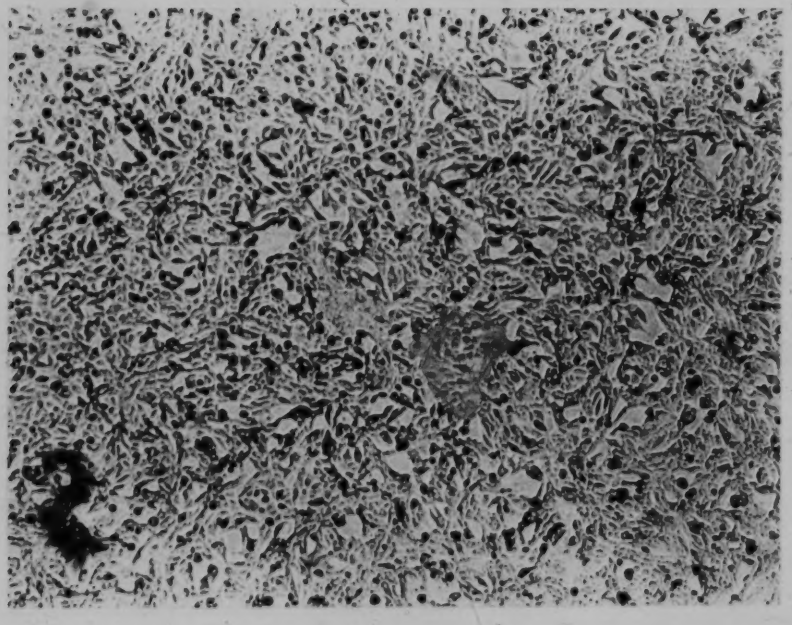
CPE and HAD observations on RP grown at 32°C

Figure 4a.

A photograph of the CPE seen at 40 h pi illustrating early cell fusion (center of photograph). This and the following photographs recording the amount of CPE or HAD of MV-infected cells were taken using an Olympus inverted light microscope (section 2.10).
Magnification 50 X

Figure 4b.

HAD at 44 h pi illustrating the adsorption of a few erythrocytes onto the syncytial surface.
Magnification 80 X .



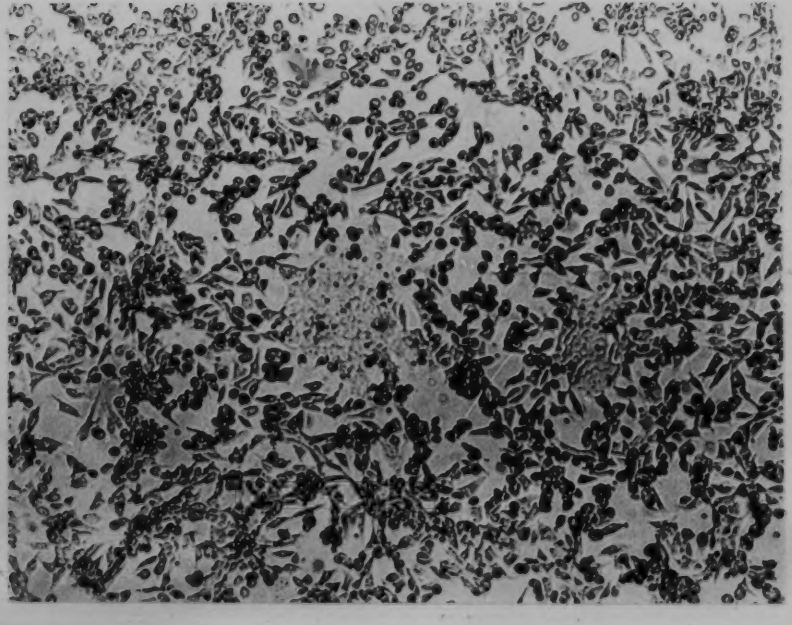
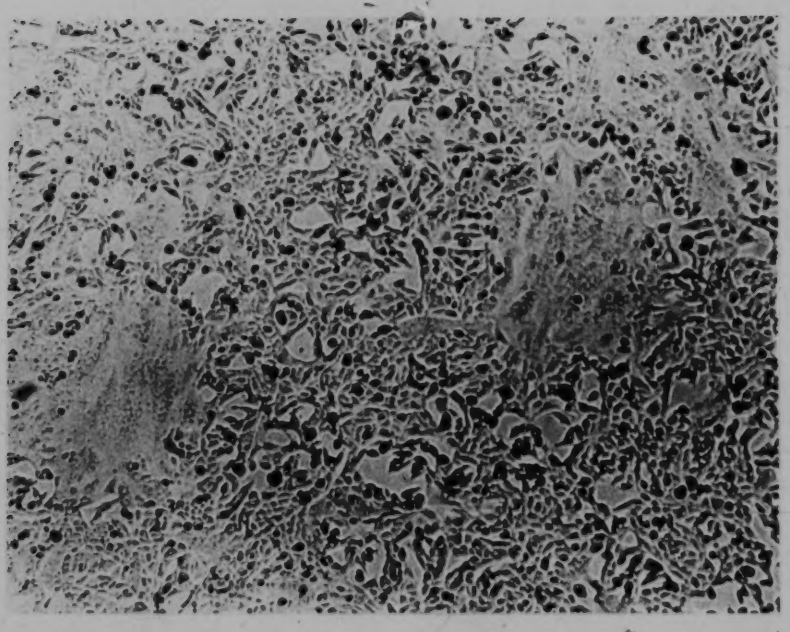


Figure 4c.

CPE (2+) seen 60 h pi in RP-infected cells at 32°C.

Figure 4d.

HAD (2+) at 60 h pi showing erythrocytes attached to approximately 50% of the syncytial area.



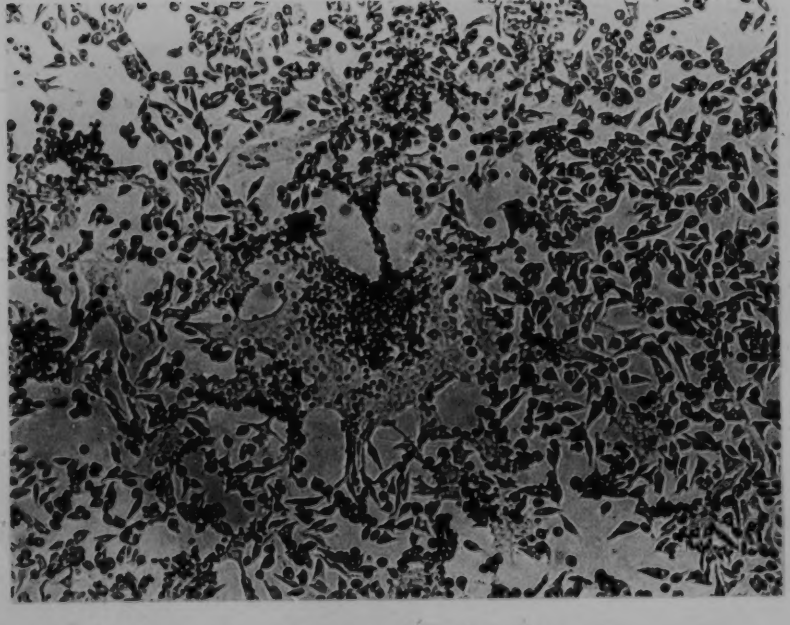
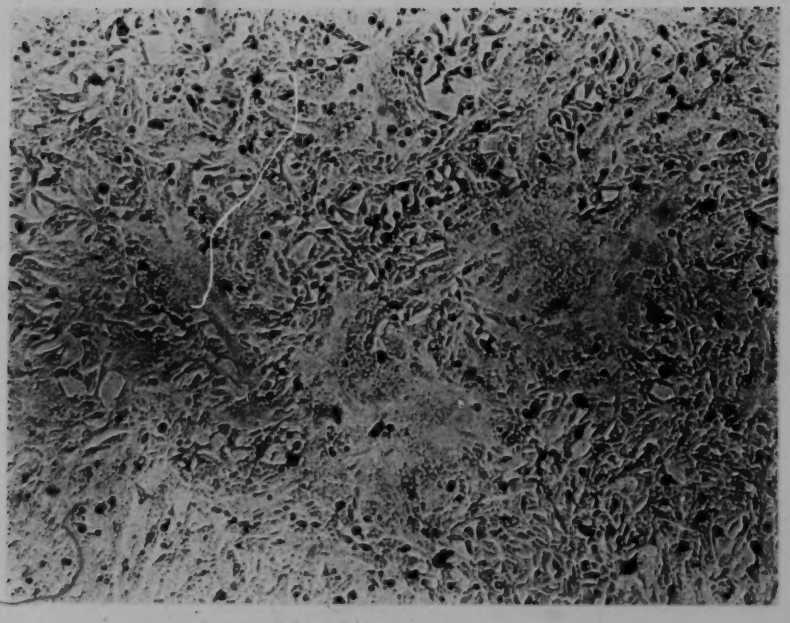


Figure 4e.

Cell fusion (3+) seen at 72 h pi.

Figure 4f.

HAD (3+) at 72 h pi showing erythrocytes attached to 50-75% of the syncytial area.



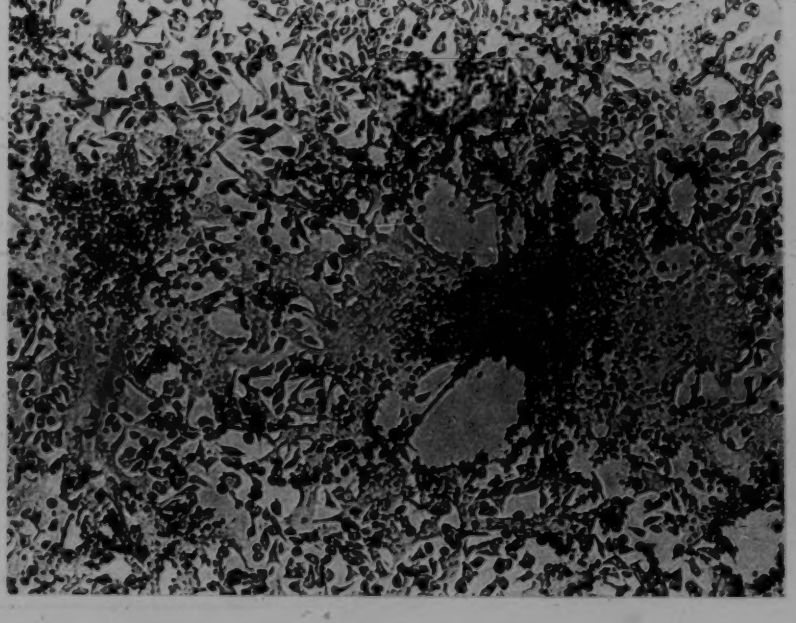
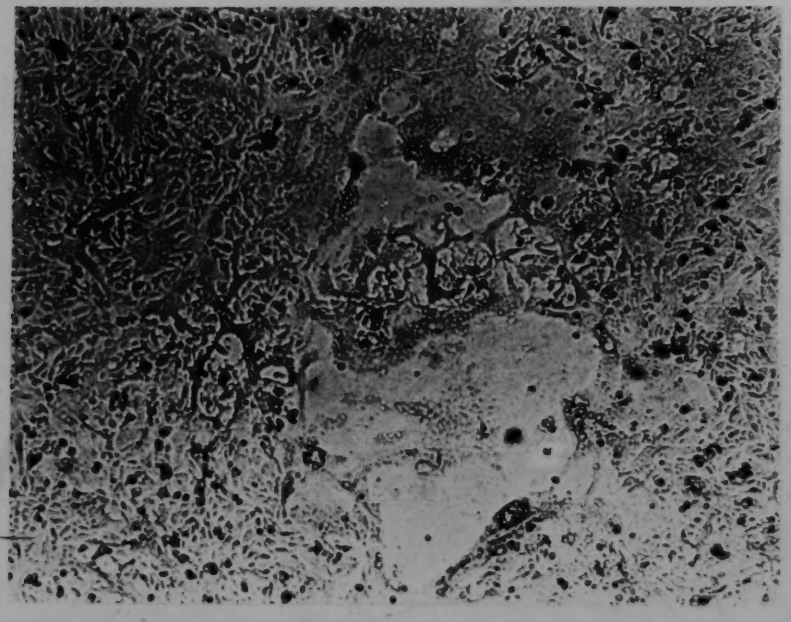


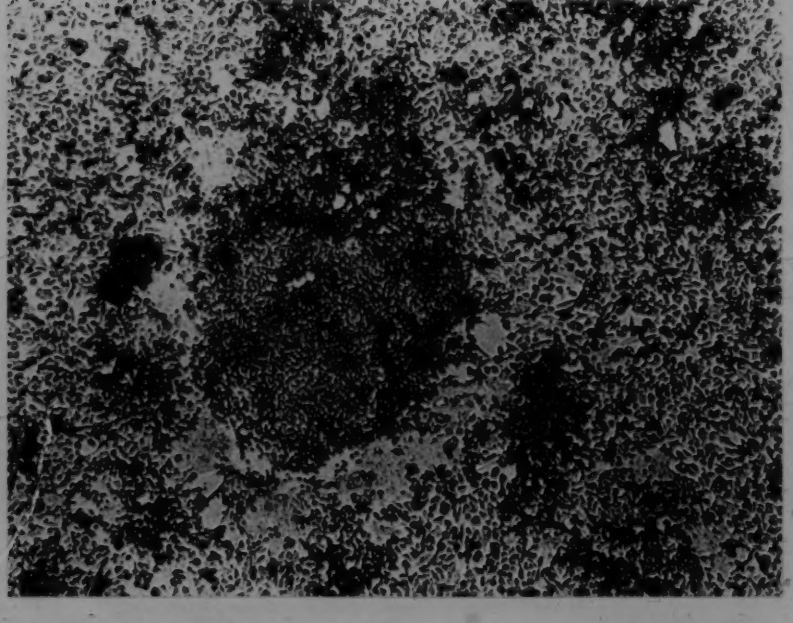
Figure 4g.

Extensive CPE (4+) at 96 h pi.

Figure 4h.

HAD (4+) at 96 h pi has attachment of erythrocytes to 75-100% of the syncytial area.





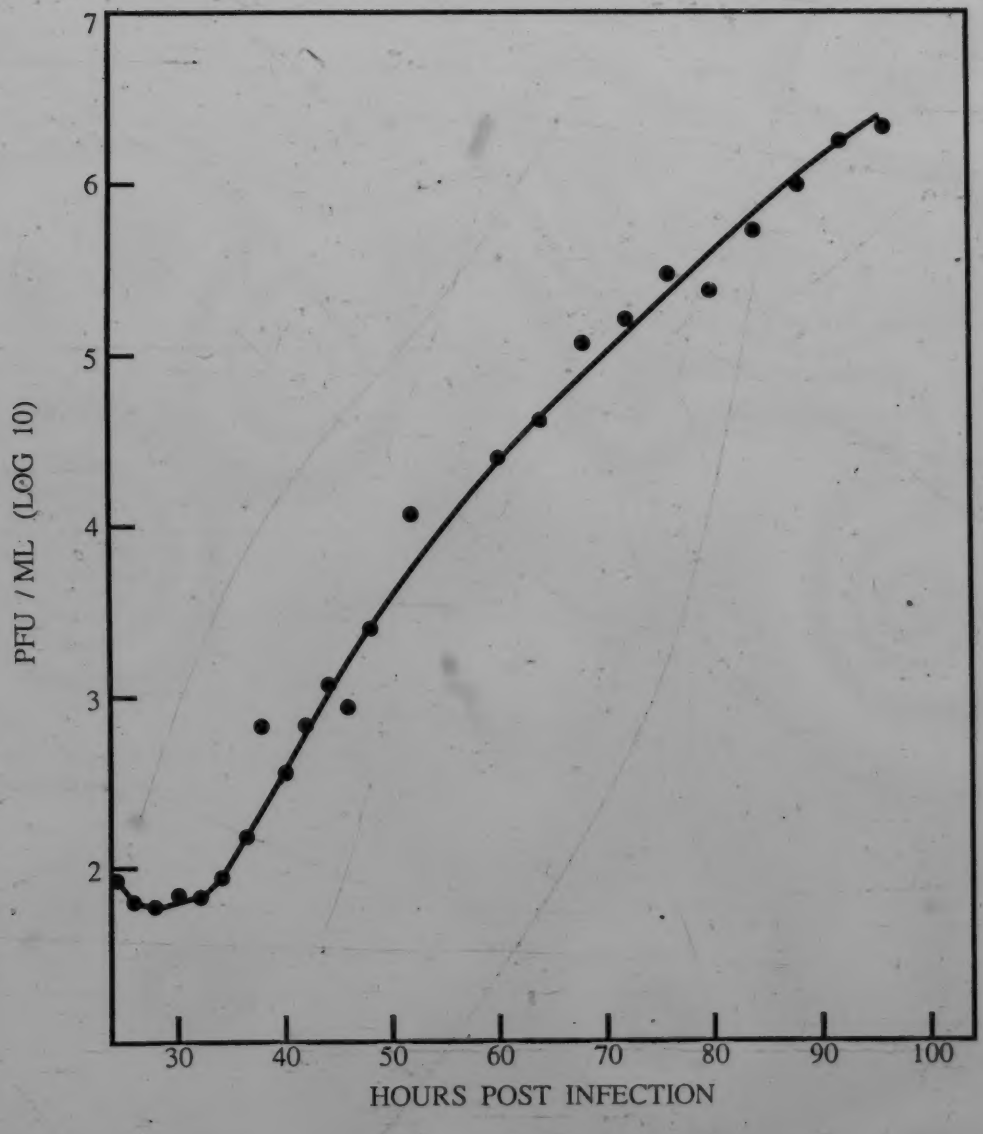
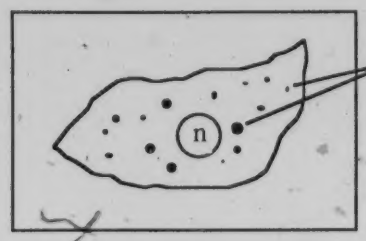


Figure 5

Production of extracellular virus by RP infected cells at 32°C

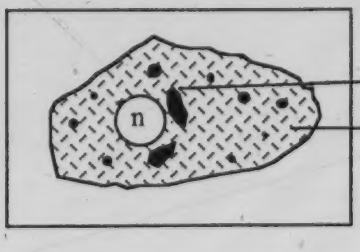
Figure 6

Progression of immunofluorescent staining obtained with NP and P protein monoclonal antibodies



dots of various sizes

refer to Fig. 9b

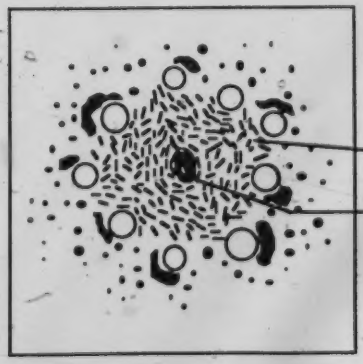


dots

globules

flecks

refer to Fig. 9c



dots/globules

flecks

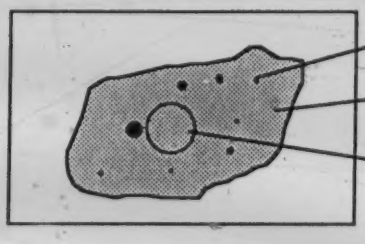
fibrous

fibrous inclusion

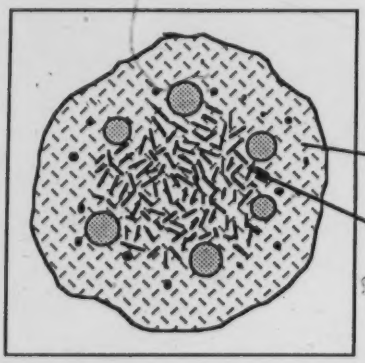
refer to Fig. 10d

Figure 7

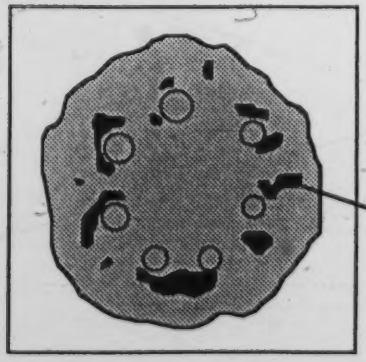
Progression of immunofluorescent staining obtained with M monoclonal antibody



refer to Fig. 11b



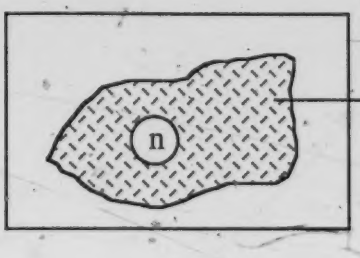
refer to Fig. 11c



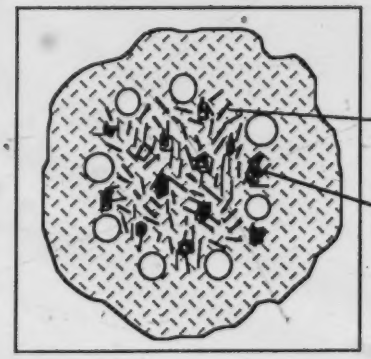
refer to Fig. 11e

Figure 8

Progression of immunofluorescent staining obtained with H and F monoclonal antibodies



refer to Fig. 12b



refer to Fig. 13d

Figure 9.

Immunofluorescent staining of the NP protein in Vero cells infected with RP at 32°C

Figure 9a.

A photograph of a RP-infected cell fluorescing after immunofluorescent staining with the NP protein monoclonal antibody. Pin point dots were detected in the cytoplasm at 6 h pi. This and the following photographs of IF staining were taken using a Leitz incident light fluorescent microscope (section 2.7 and 2.10).
Magnification 512 X

Figure 9b.

Fluorescent dots in a variety of sizes detected in the cytoplasm at 14 h pi.

Figure 9c.

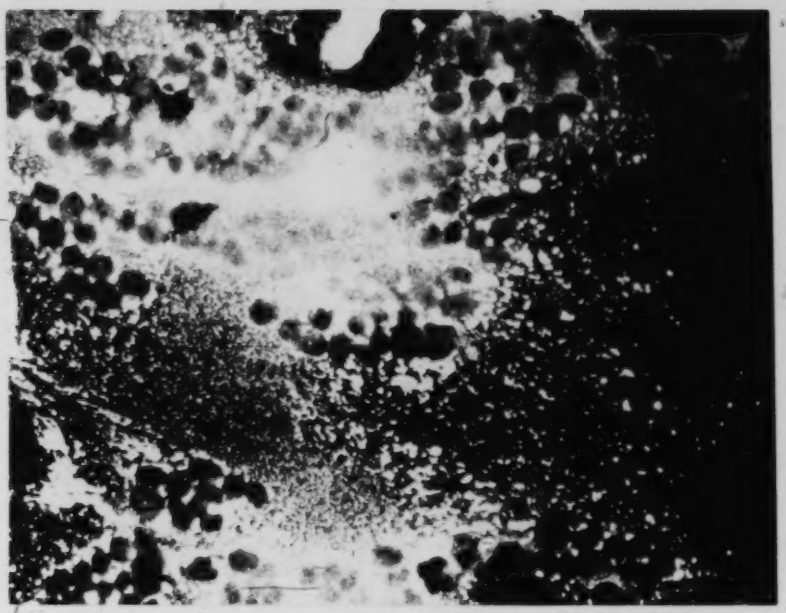
The appearance of flecks in addition to the bright dots and larger globules at 24 h pi.

Figure 9d.

NP staining at 48 h pi showing extensive cytoplasmic staining.
Magnification 320 X

Figure 9e.

NP staining at 72 h pi illustrating the fibrous type staining in the center of syncytia, perinuclear globular inclusions, and flecks and dots towards the periphery of the syncytia.



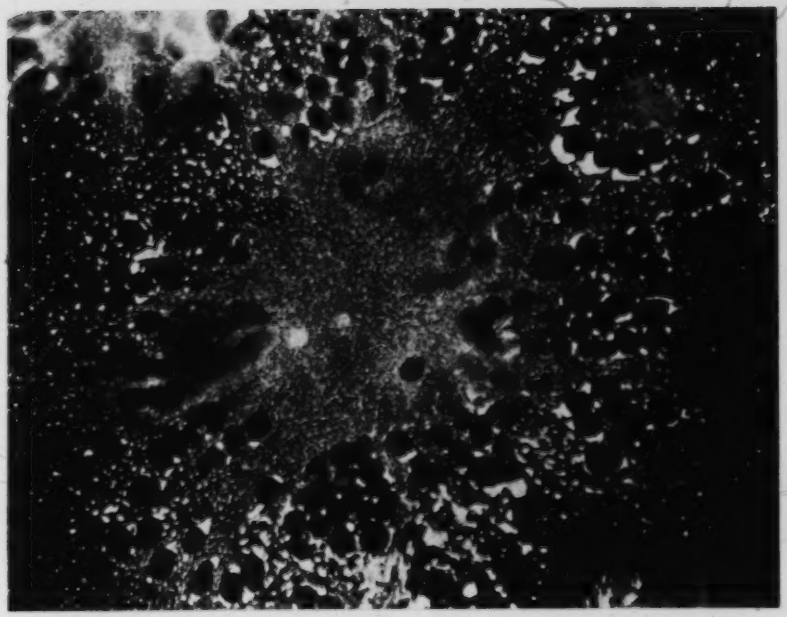


Figure 10.

Immunofluorescent staining of the P protein in cells infected with RP at 32°C

Figure 10a.

Cytoplasmic dots of P protein seen at 10 h pi.

Figure 10b.

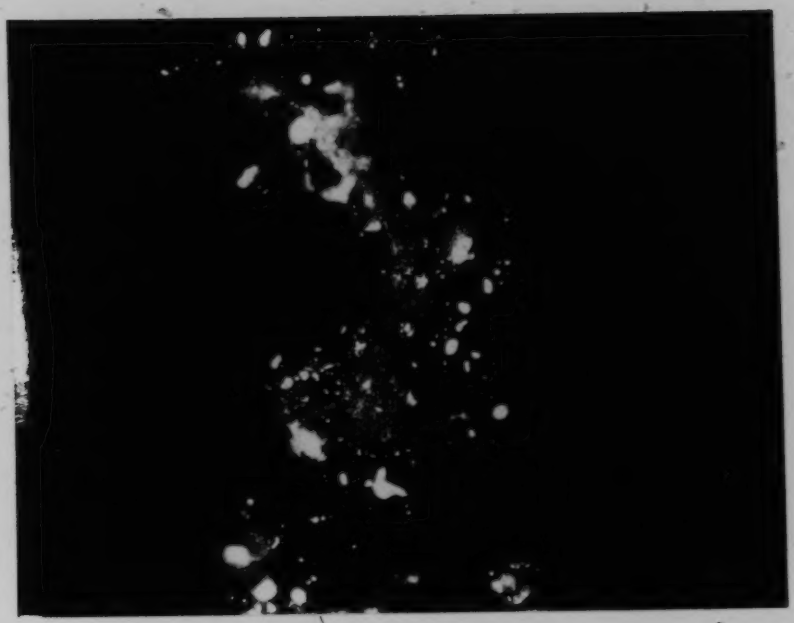
Flecks, bright dots and large globules of P protein seen at 24 h pi.

Figure 10c.

Appearance of P protein at 48 h pi showing flecks, bright dots and globules and some fibrous material.

Figure 10d.

A good example of the different types of cytoplasmic staining seen with P protein at 72 h pi. Present are dots/globules, flecks, fibrous material (f) and fibrous inclusions (i).



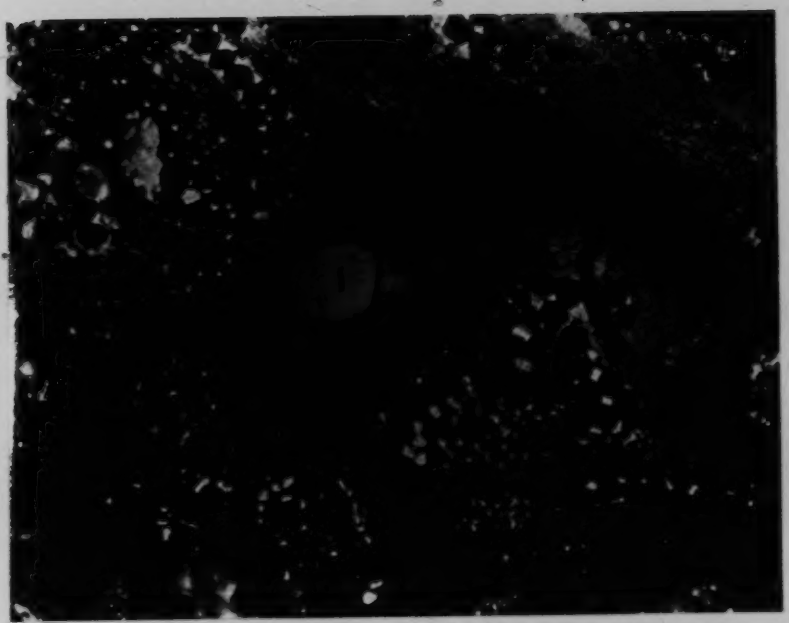


Figure 11.

Immunofluorescent staining of the M protein in cells infected with RP at 32°C

Figure 11a.

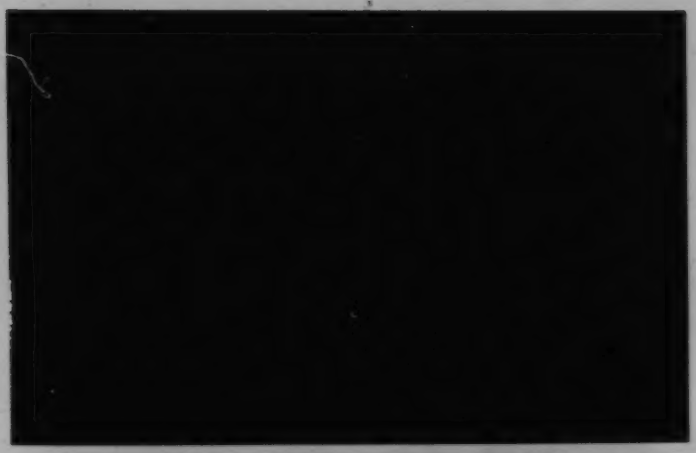
A faintly stained cell at 18 h pi showing pale diffuse nuclear staining, faint cytoplasmic staining with a few dots and globules of M protein.

Figure 11b.

An infected cell at 24 h pi showing the same features seen at 18 h pi but more intensely.

Figure 11c.

A small syncytia at 48 h pi characterized by flecks, fibrous material, a few globules and pale nuclear staining.



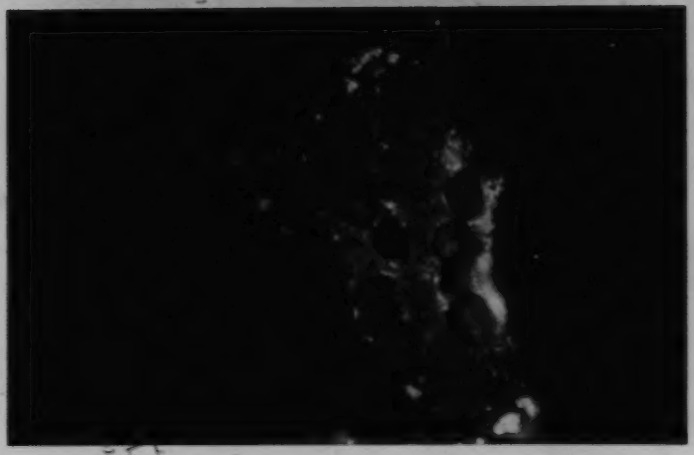


Figure 11d.

A brightly-staining flecked and fibrous type of syncytia seen at 72 h pi.

Figure 11e.

A pale-staining type of syncytia at 80 h pi characterized by a pale homogeneous cytoplasm containing weakly staining globular perinuclear inclusions and diffuse nuclear staining.

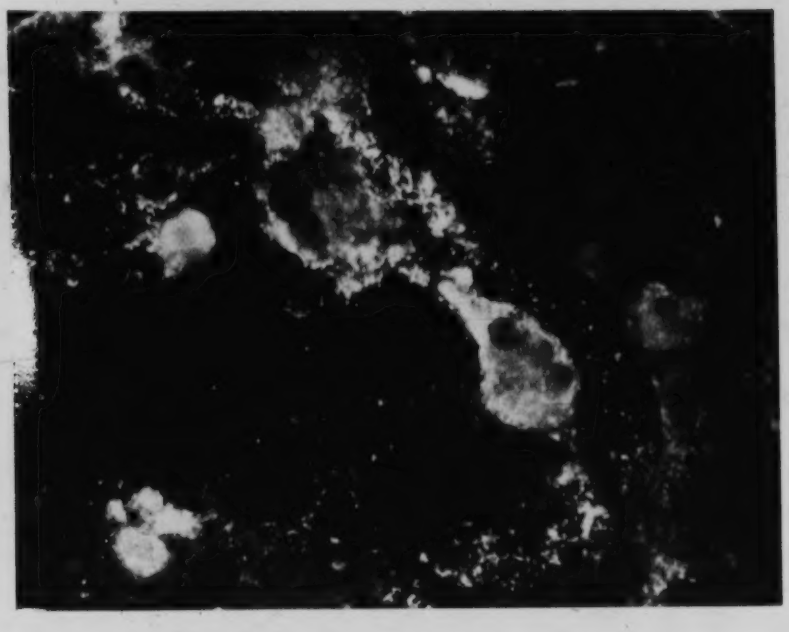


Figure 12.

Immunofluorescent staining of the H protein in cells infected with RP at 32°C

Figure 12a.

An infected cell (20 h pi) showing diffuse homogeneous cytoplasmic staining, pale flecks and no nuclear staining with the anti-H monoclonal antibody.

Figure 12b.

Distinct flecking seen at 24 h pi.

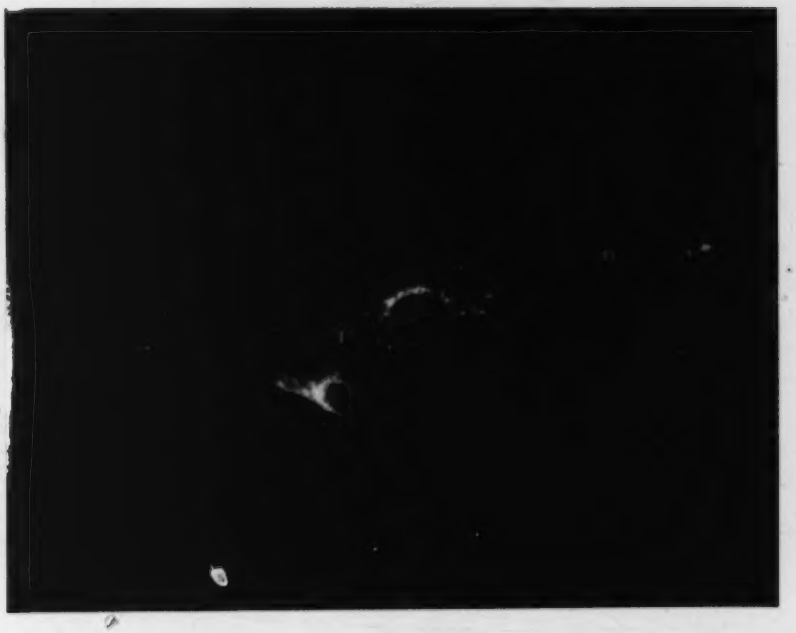


Figure 12c.

H protein at 40 h pi shows distinct flecks and fibrous material.

Figure 12d.

Fibrous clusters are a predominant feature of this syncytia at 56 h pi.

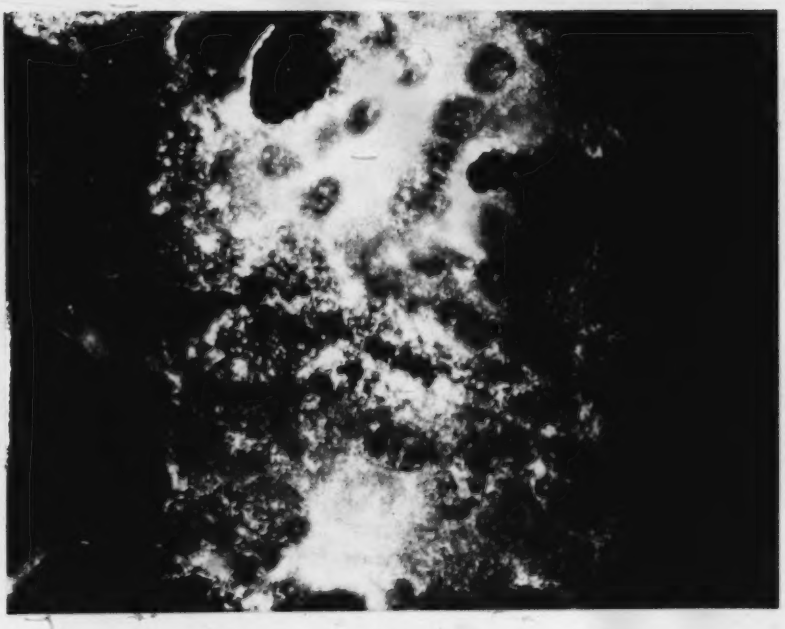


Figure 13.

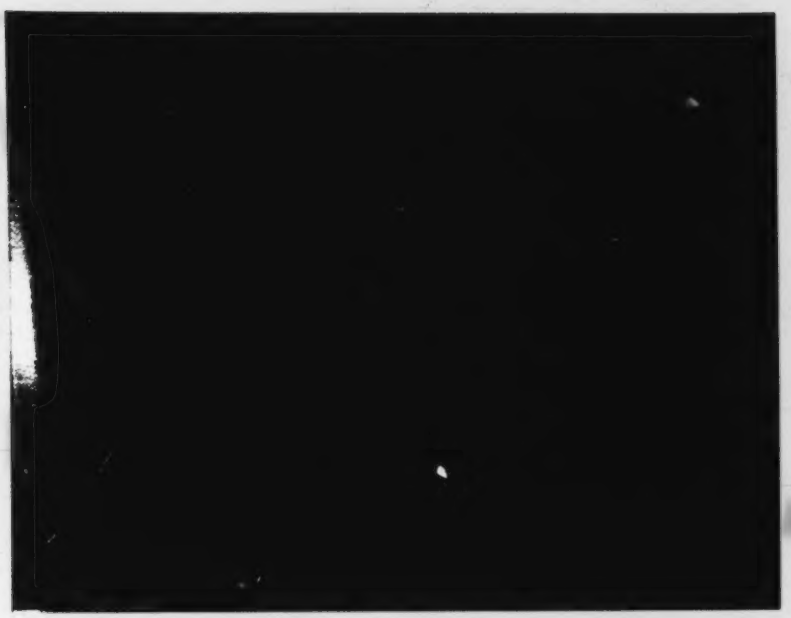
Immunofluorescent staining of the F protein in cells infected with RP at 32°C

Figure 13a.

Pale cytoplasmic flecks of F protein detected at 21 h pi.

Figure 13b.

Pale flecking at 24 h pi.



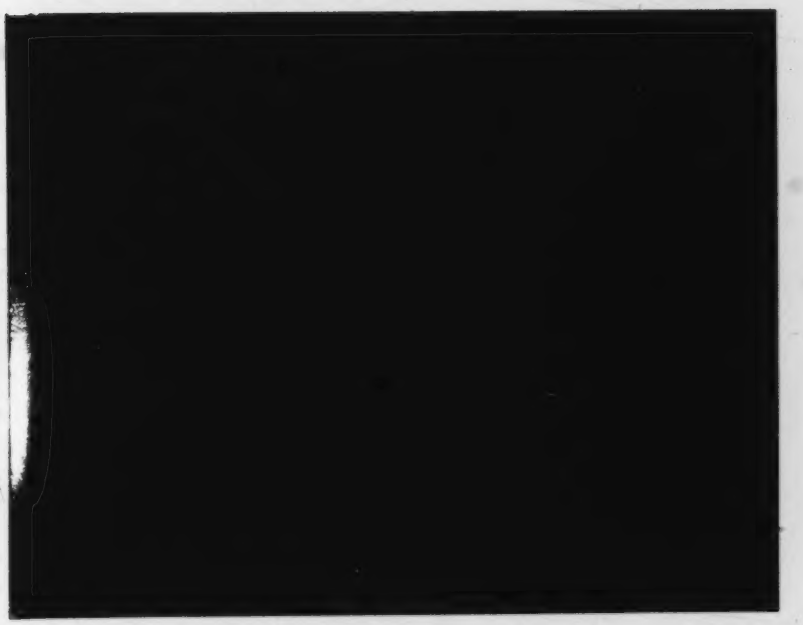
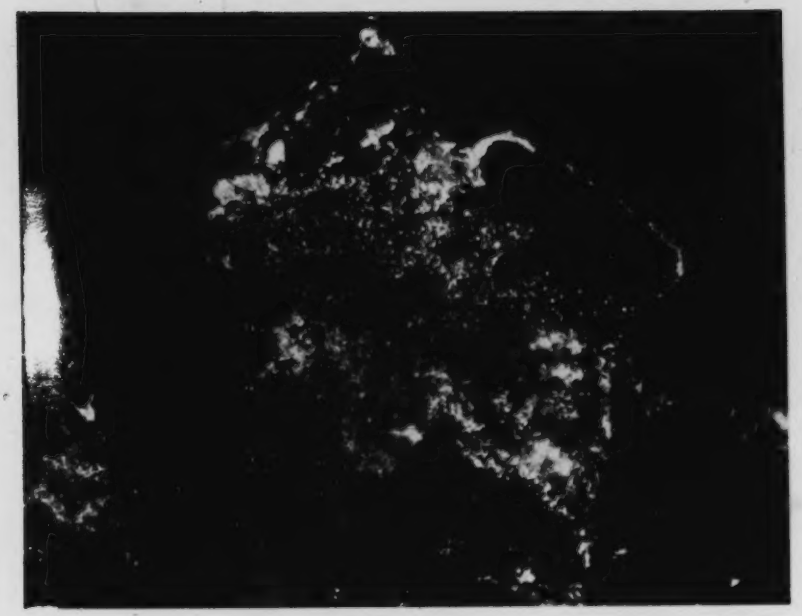


Figure 13c.

F protein stained at 48 h pi illustrating the bright flecks, fibrous material and some fibrous clusters

Figure 13d.

A good illustration of the F protein fibrous clusters (c) and fibrous material (f) seen at 72 h pi.



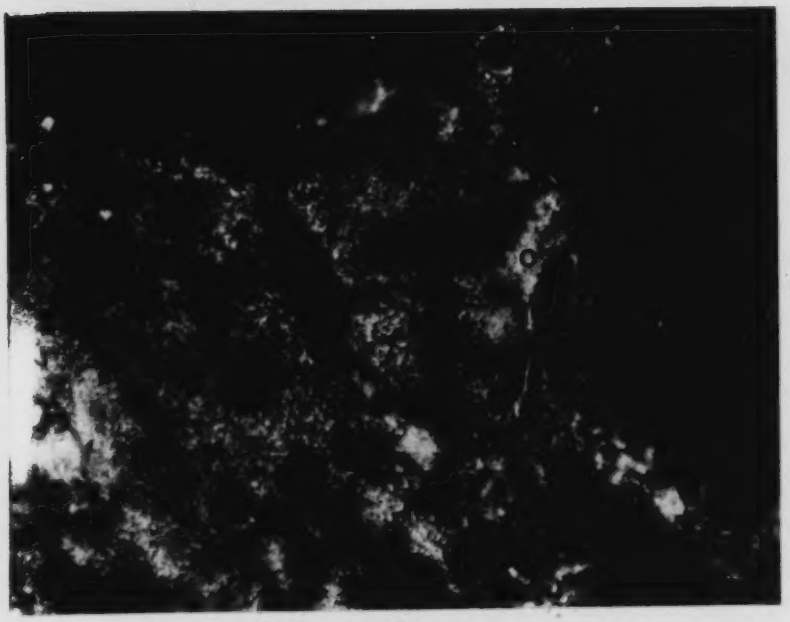


Figure 14.

Electron micrographs of cells infected with RP at 32°C

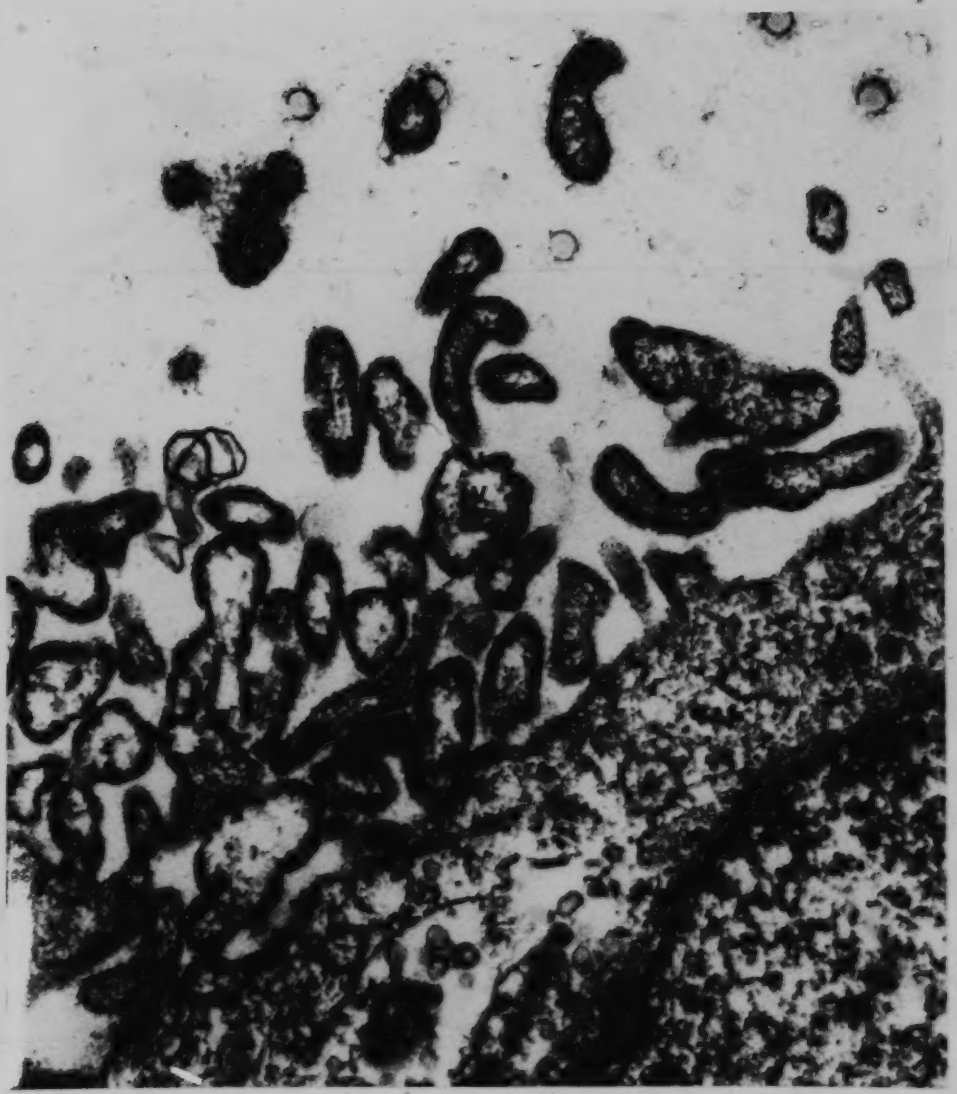
Figure 14a.

An electron micrograph of a RP-infected cell at 96 h pi illustrating a cytoplasmic inclusion of NC material. Refer to sections 2.8 and 2.9 for the methods used.
Magnification 25,500 X

NC

Figure 14b.

Extracellular virus (v) seen at 72 h pi.
Magnification 51,000 X.



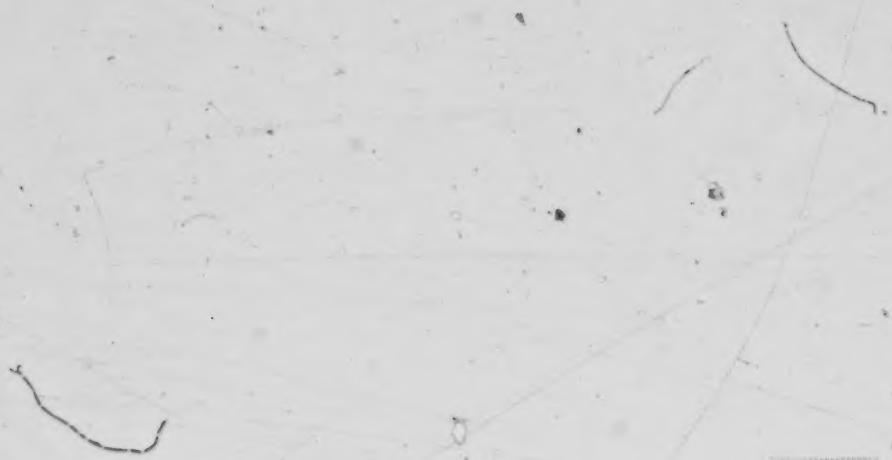


Figure 14c.

Infected cells at 96 h pi showing budding virus (bv) and simple nuclear bodies (sNB).
Magnification 25,500 X

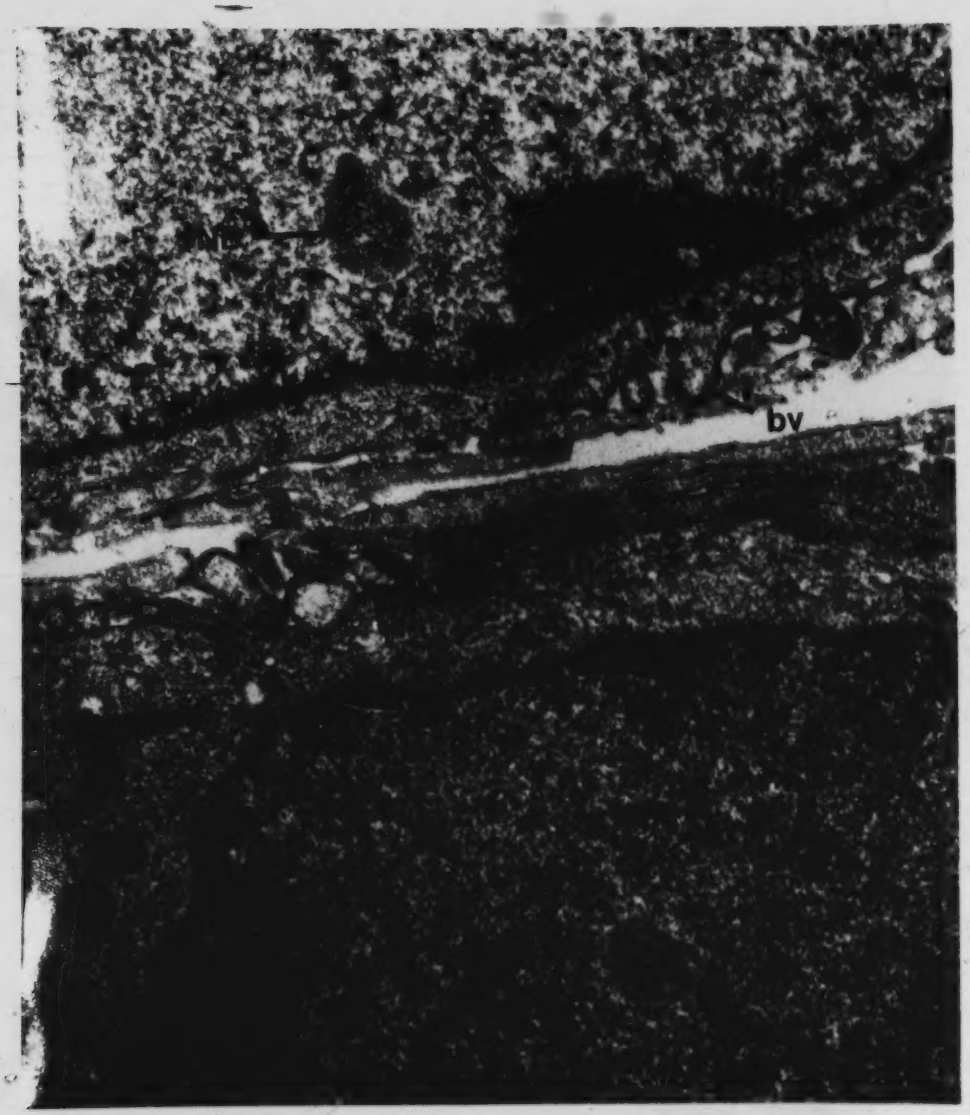

bv

Figure 15.

Immunofluorescent staining of Lec and mumps virus-infected cells with M monoclonal antibody (C1-144)

Figure 15a.

Lec-infected cells stained with the C1-144 clone showed diffuse nuclear staining.

Figure 15b.

The uninfected control cells do not stain intranuclearly with the C1-144 clone.

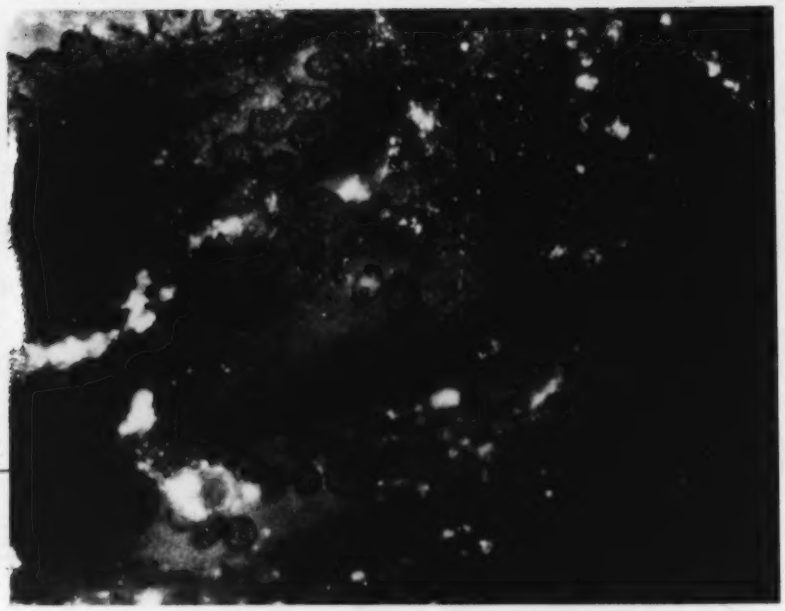


Figure 15c.

Mumps virus-infected cells do not stain with the C1-144 clone of M monoclonal antibody.

Figure 15d.

Nonstained uninfected control cells.

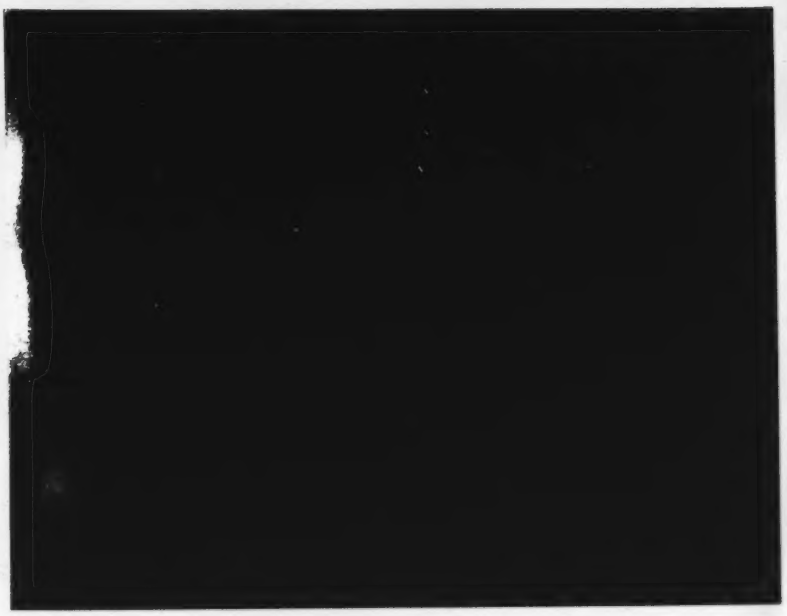


Figure 16.

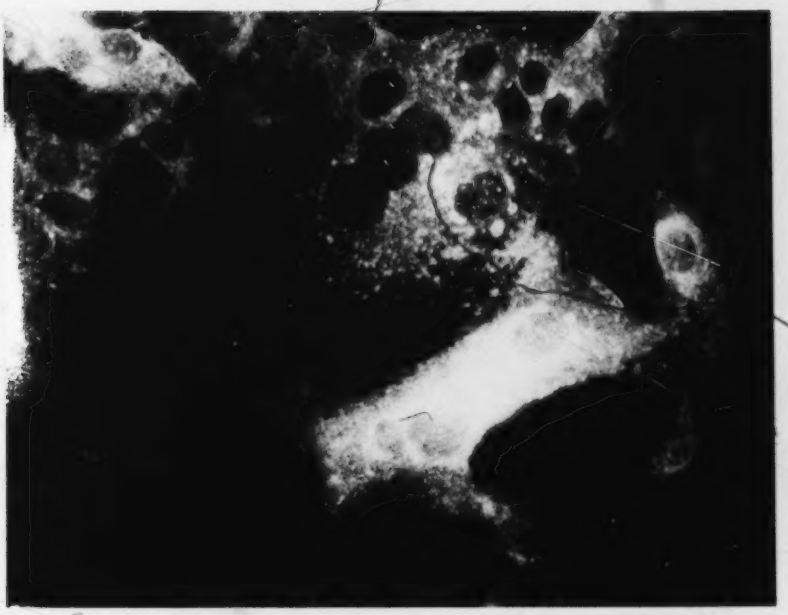
Immunofluorescent staining of Halle UP-1 cells with polyclonal wild-type antiserum (MVR-3)

Figure 16a.

Brightly fluorescing infected cells at 32°C and 51 h pi showing primarily flecks, fibrous material and fine homogeneous cytoplasmic staining.

Figure 16b.

A syncytia at 39°C and 27 h pi illustrating the pale diffuse nuclear staining, fine cytoplasmic staining as well as bright dots/globules, flecks and fibrous staining.



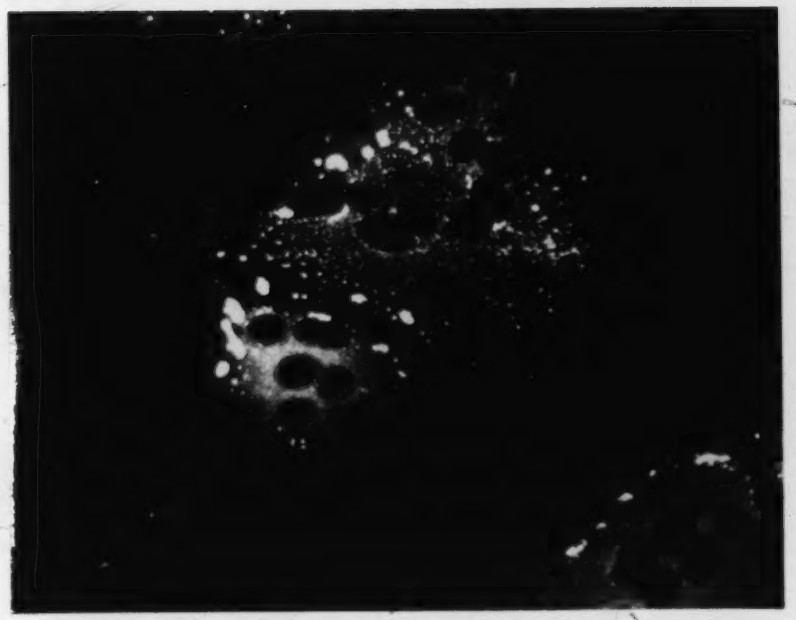


Figure 17.

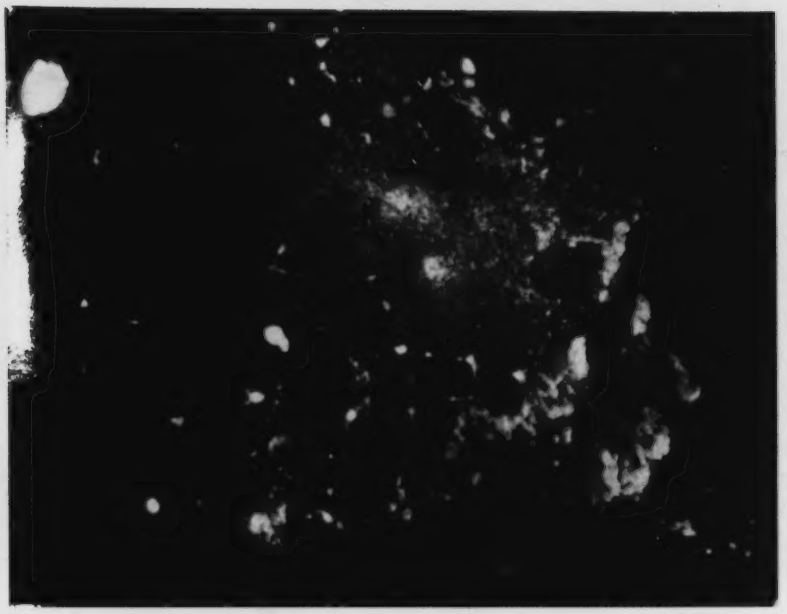
Immunofluorescent staining of Halle UP-1 cells with polyclonal nucleocapsid antiserum (MeNC-1)

Figure 17a.

Infected cells at 32°C and 51 h pi demonstrating diffuse and dotted nuclear staining and intense cytoplasmic staining.

Figure 17b.

A large syncytia at 39°C and 27 h pi illustrating nuclear diffuse staining and dots as well as bright cytoplasmic globular inclusions, dots and areas of only fine homogeneous staining.



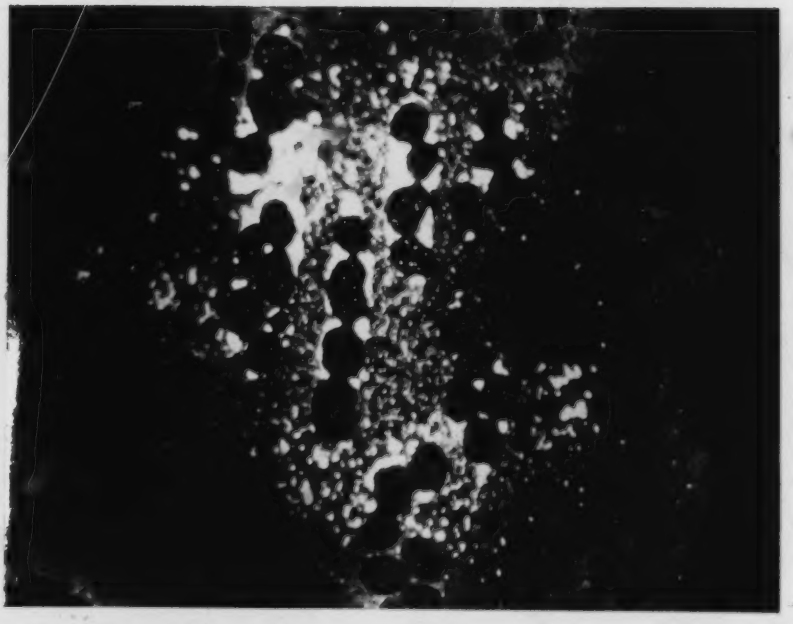


Figure 18.

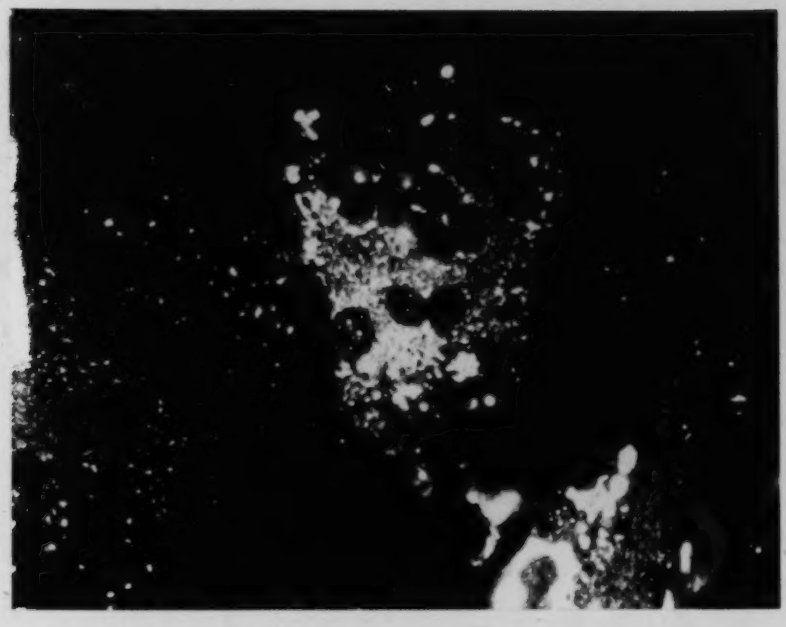
Immunofluorescent staining of Halle UP-1 cells with NP monoclonal antibody (16AC5)

Figure 18a.

The typical staining seen 51 h pi at 32°C with the flecks, fibrous material, dots and globules located cytoplasmically and the occasional nuclei with dots.

Figure 18b.

A similar-sized syncytia 27 h pi at 39°C showing many bright dots and globules and some flecking but less fibrous material as compared to fig. 18a.



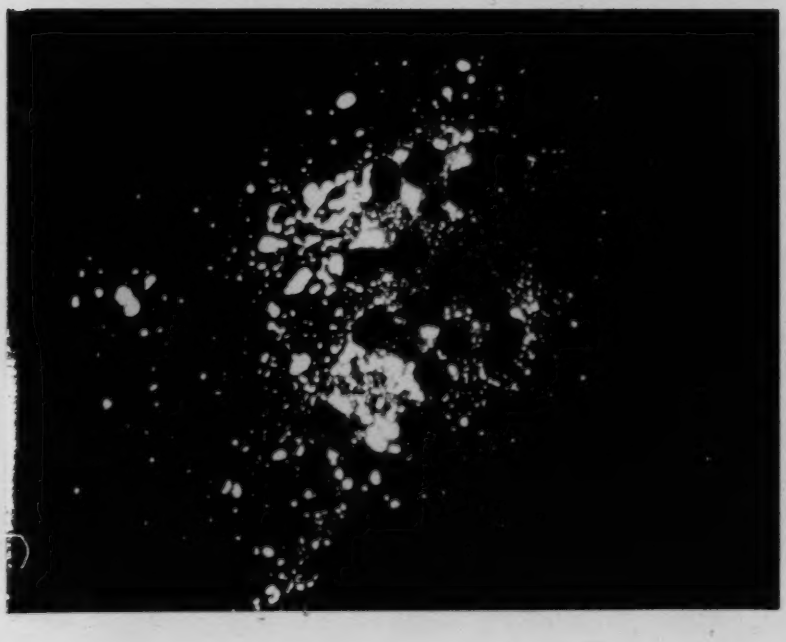
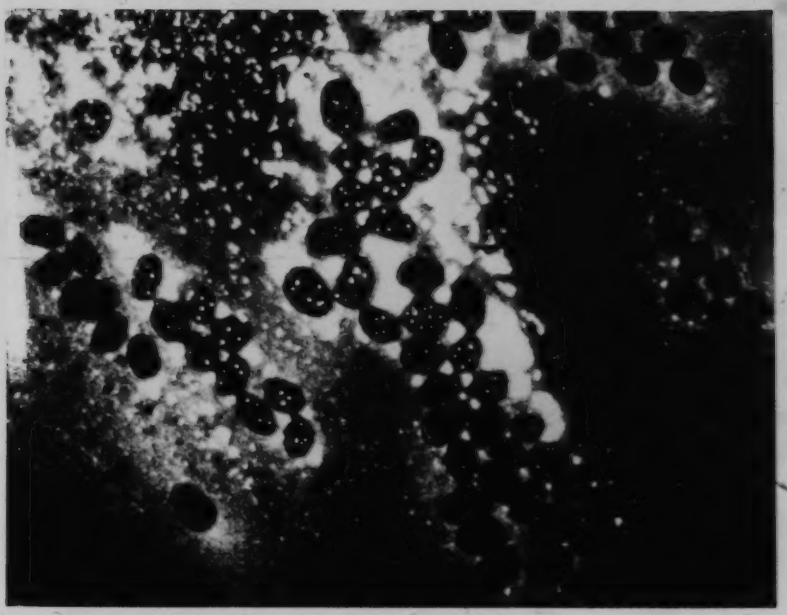


Figure 18c.

Distinct nuclear dots seen at 42 h pi and at 39°C.

Figure 18d.

A good example of the type of syncytia lacking flecks and fibrous material. This syncytia was observed 3 d pi at 39°C.



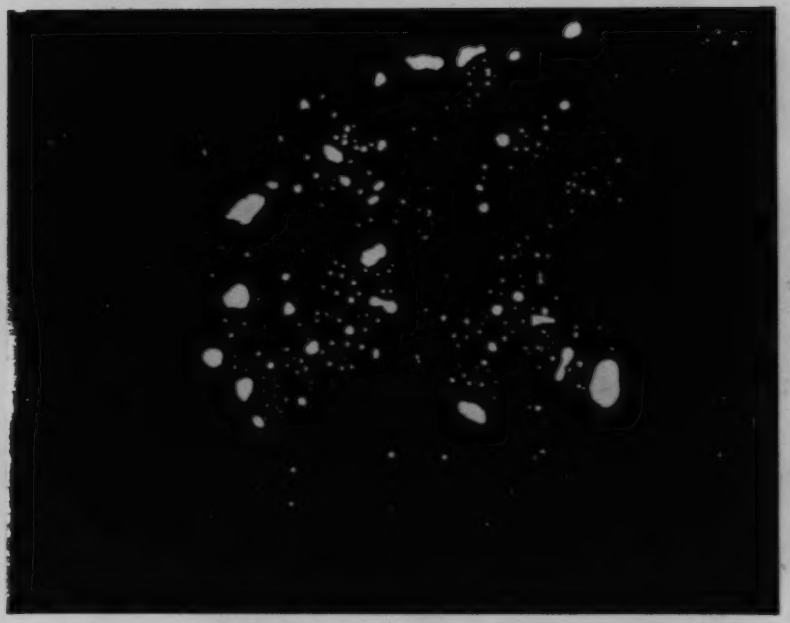


Figure 19.

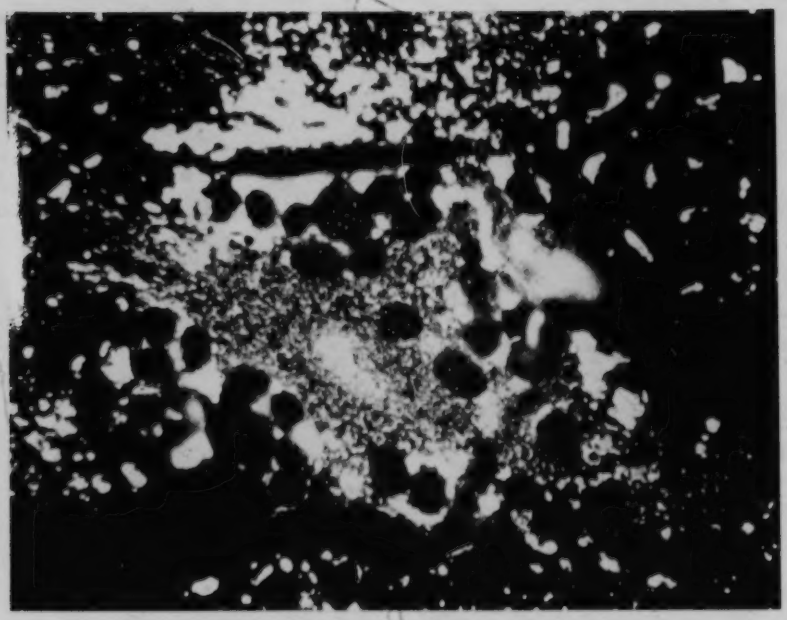
Immunofluorescent staining of Halle UP-1 cells with P monoclonal antibody (C1-105)

Figure 19a.

An intensely stained syncytia observed 51 h pi at 32°C showing types 1, 2, 3 and 4 cytoplasmic staining and a few nuclei with dots.

Figure 19b.

A less-flecked and less-fibrous type of syncytia at 42 h pi and 39°C showing tiny intranuclear fluorescing dots.



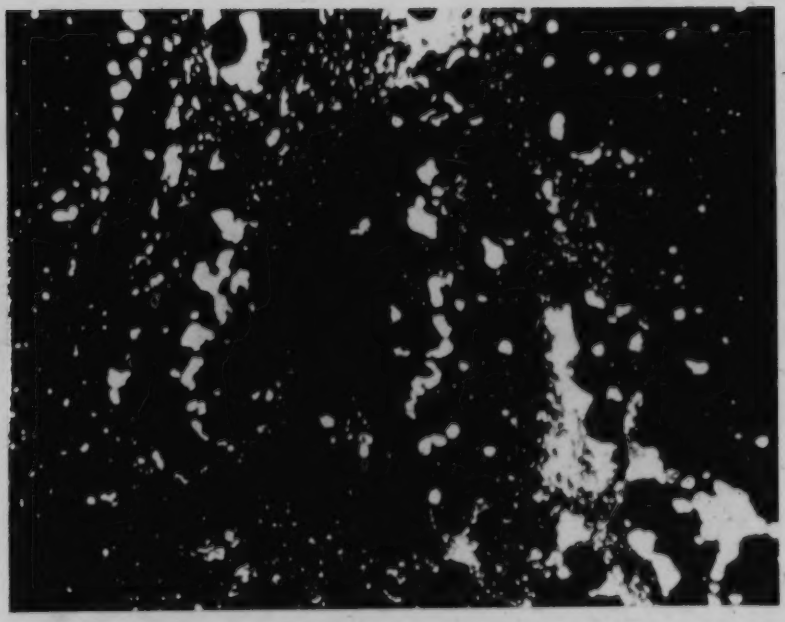


Figure 20.

Immunofluorescent staining of Halle UP-1 cells with CDV P protein monoclonal antibody

Figure 20a.

Distinct nuclear dots but very weak cytoplasmic staining observed 51 h pi at 32°C.

Figure 20b.

A large syncytia demonstrating a nonstaining periphery and primarily type 1 cytoplasmic staining and type 7n nuclear staining at 42 h pi and 39°C.

Figure 21.

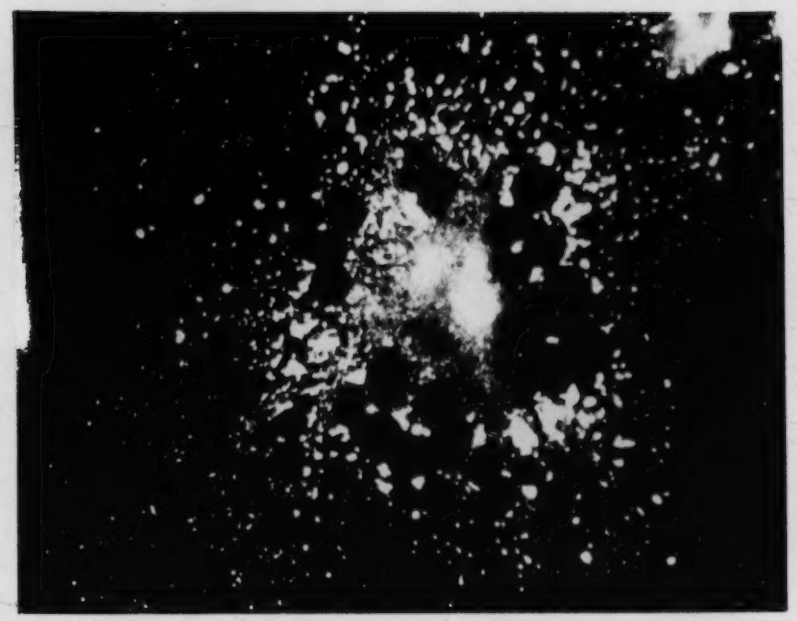
Immunofluorescent staining of Halle UP-1 cells with P monoclonal antibody (16AF10 or 16BD3)

Figure 21a.

Typical syncytia at 32°C and 51 h pi showing types 1, 2, 3 and 4 cytoplasmic staining but no nuclear staining.

Figure 21b.

Typical syncytia at 39°C, 27 h pi characterized by less-fibrous and less-flecked type of staining.



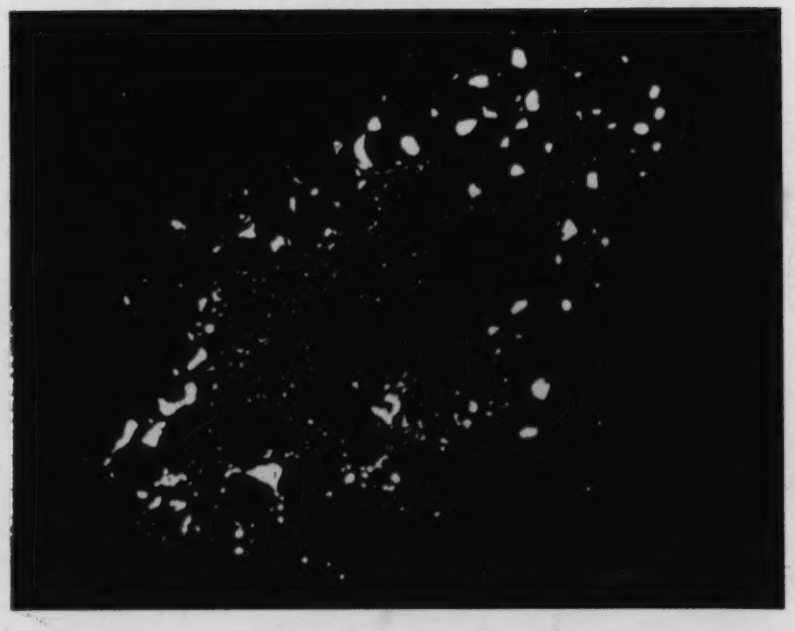


Figure 22.

Immunofluorescent staining of Halle UP-1 cells with M monoclonal antibody (C1-144)

Figure 22a.

A brightly stained syncytia at 51 h pi and 32°C demonstrating the typical appearance at this temperature. The nuclei are stained in addition to the cytoplasmic staining (types 2,3,4 and 6).

Figure 22b.

This large syncytia at 27 h pi and 39°C is like the 32°C type of syncytia without the flecks and fibrous material.

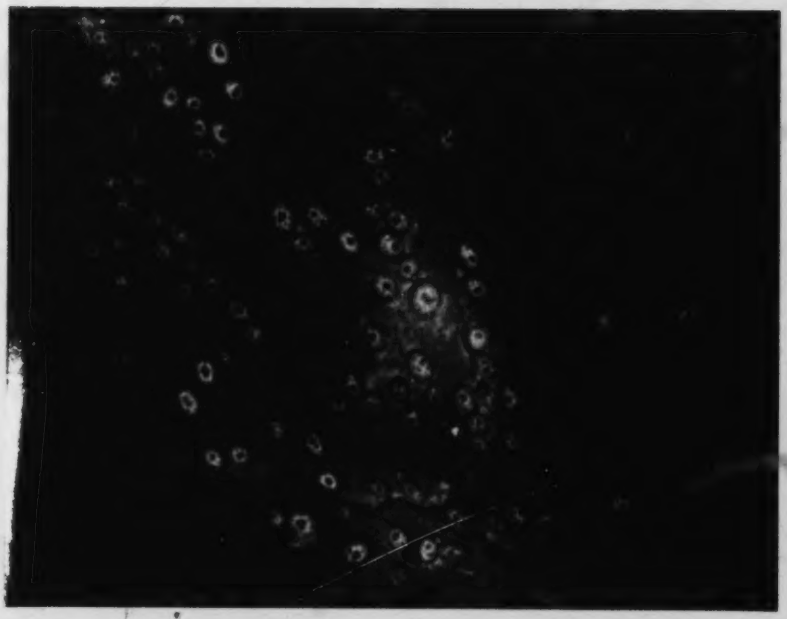


Figure 22c.

A good example of the pale perinuclear inclusions that are seen in 39°C syncytia at 42 h pi. Also the nuclear staining is quite strong and small fluorescing dots appear to be forming in the nuclei (arrow).

Figure 23.

Immunofluorescent staining of Halle UP-1 cells with H monoclonal antibody (C1-15)

Figure 23a.

The brightly stained type of syncytia seen at 32°C and 51 h pi characterized by flecks, fibrous material and fibrous clusters.

Figure 23b.

A syncytia at 39°C and 27 h pi illustrating very pale flecking.

Figure 24.

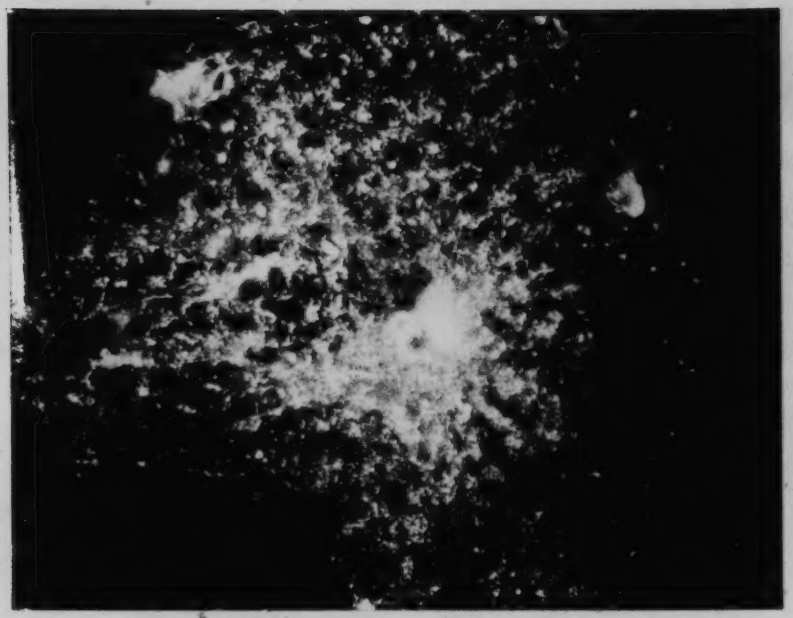
Immunofluorescent staining of H[illegible]e UP-1 cells with F monoclonal antibody (19BG4)

Figure 24a.

A typical, brightly stained syncytia at 32°C and 51 h pi showing flecks, fibrous material and fibrous clusters.

Figure 24b.

A typical weakly stained syncytia at 39°C and 27 h pi.



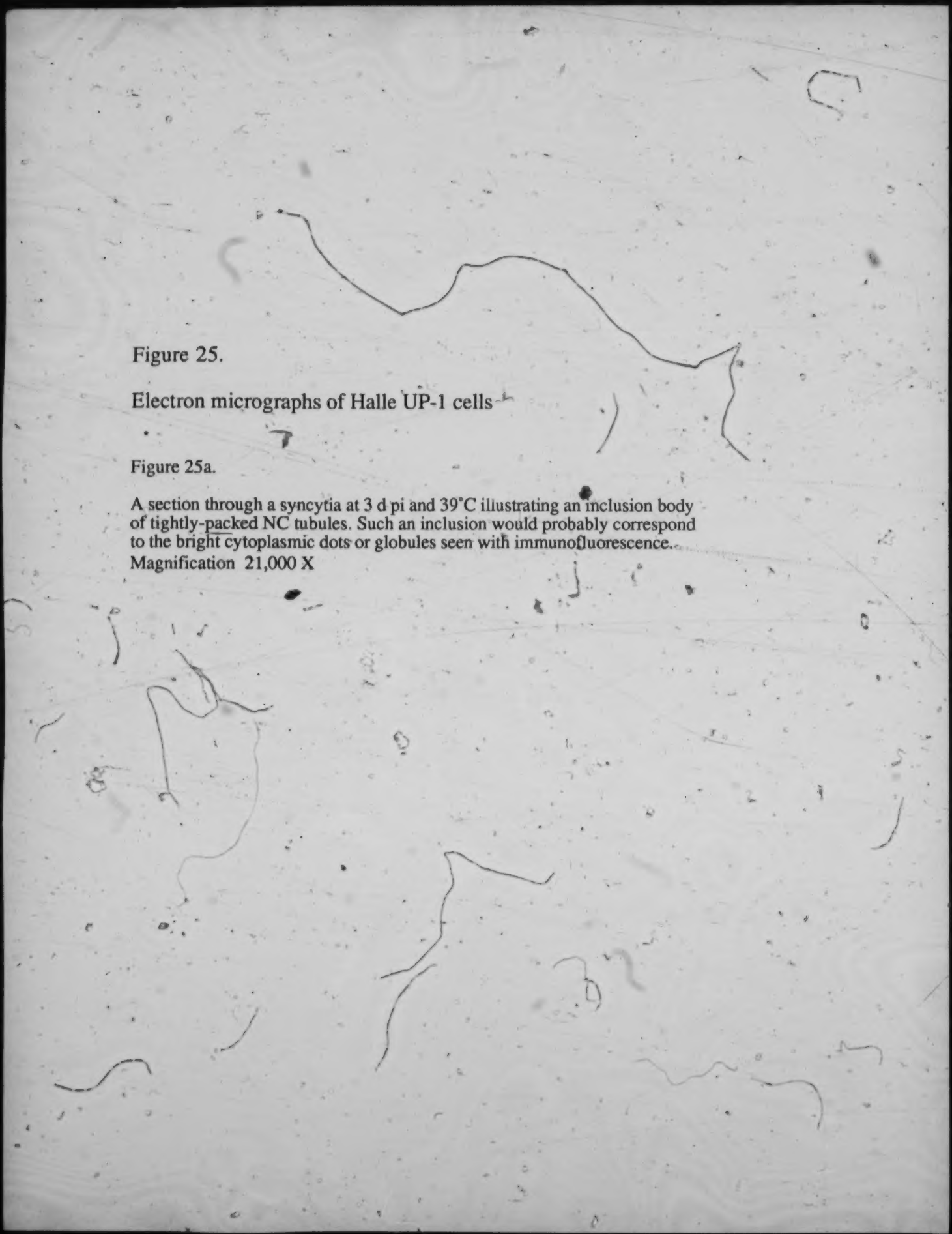


Figure 25.

Electron micrographs of Halle UP-1 cells

Figure 25a.

A section through a syncytia at 3 d pi and 39°C illustrating an inclusion body of tightly-packed NC tubules. Such an inclusion would probably correspond to the bright cytoplasmic dots or globules seen with immunofluorescence. Magnification 21,000 X

Figure 25b.

An infected cell 3 d pi in the UP-1(39) culture.
The infection process has advanced in this cell to the point where the entire cytoplasm is filled by nucleocapsid material.
Magnification 32,500 X

Figure 25c.

This micrograph of a UP-1(39) infected cell at 3 d pi illustrates three simple NB (sNB) in close proximity to one another and the beginning of nucleolar destruction (nd).
Magnification 41,500 X

Figure 26.

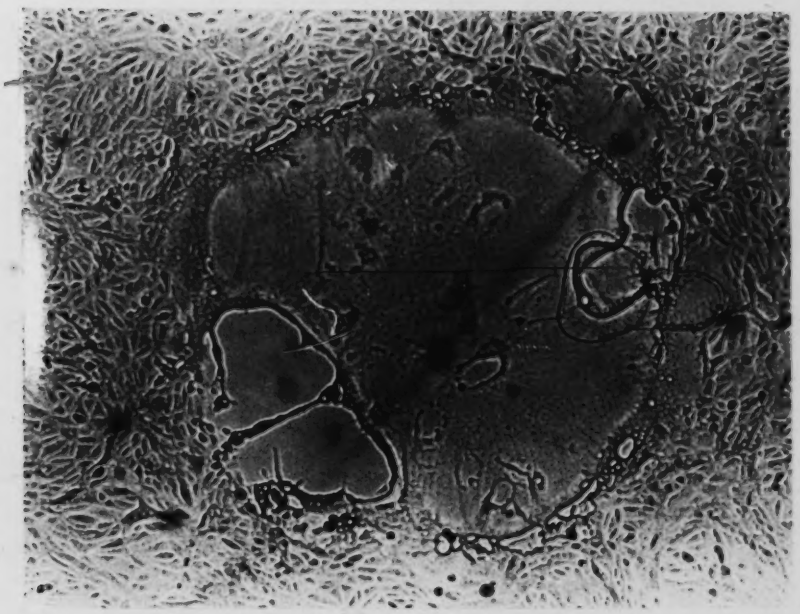
CPE and HAD observations on Halle UP-2 cells

Figure 26a.

An active syncytia seen 3 d pi at 39°C.
Magnification 80 X

Figure 26b.

Positive HAD on a syncytia at 39°C.



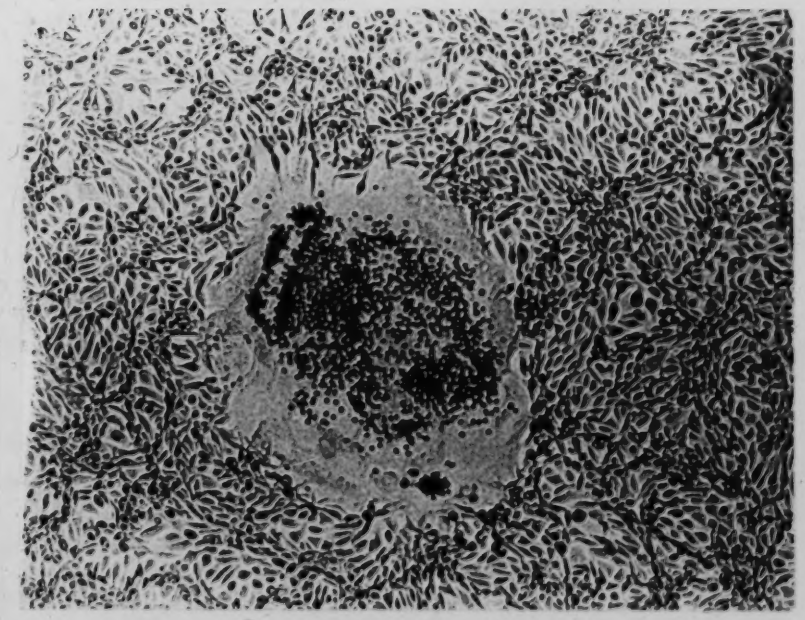
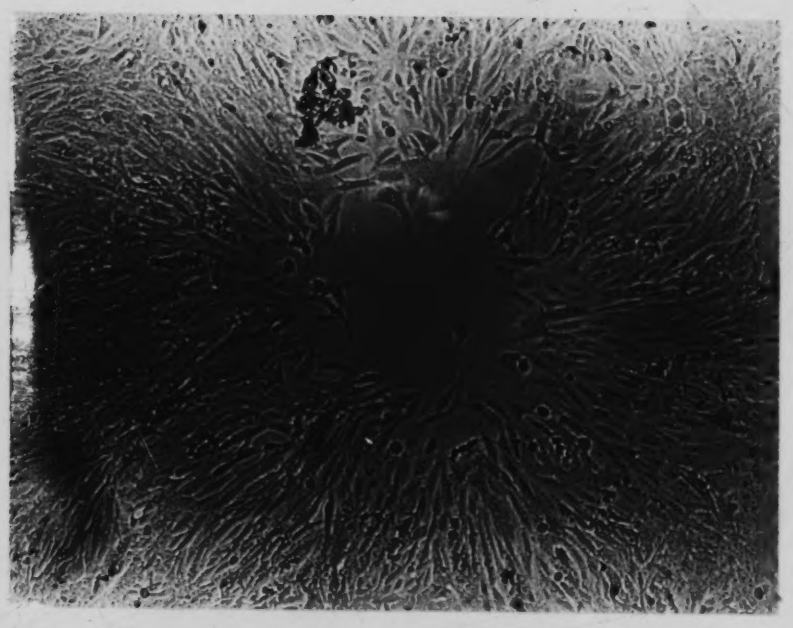


Figure 26c.

A healing syncytial hole observed 5 d pi at 39°C.

Figure 26d.

Weak HAD observed on a syncytial hole at 39°C.



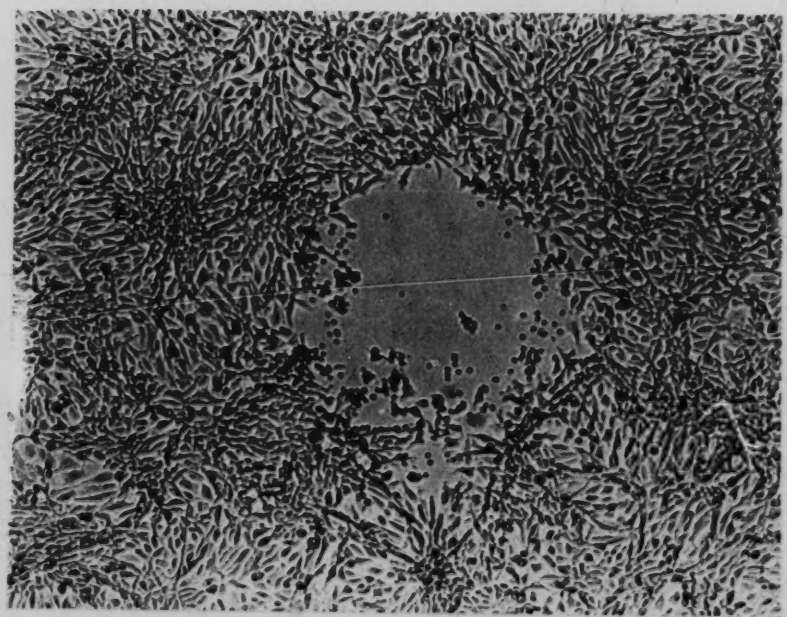
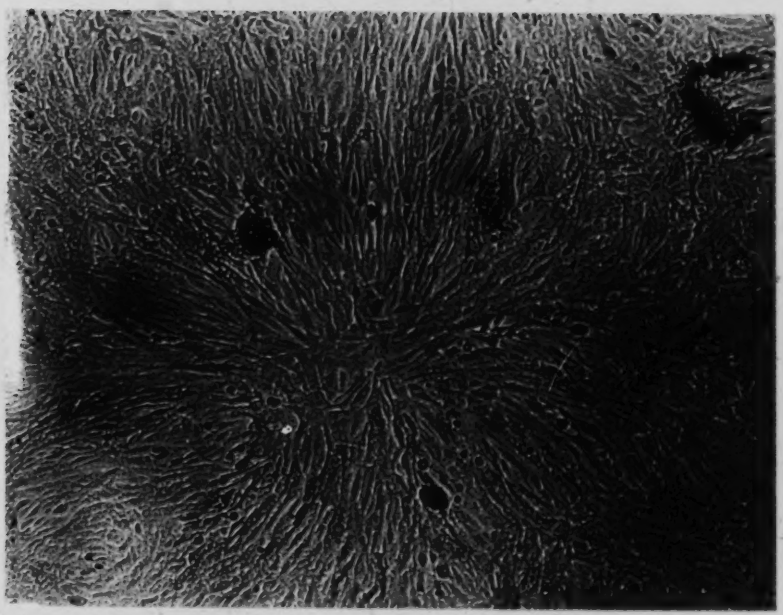


Figure 26e.

A completely healed hole seen 7 d pi at 39°C.

Figure 26f.

A healed plaque showing negative HAD.



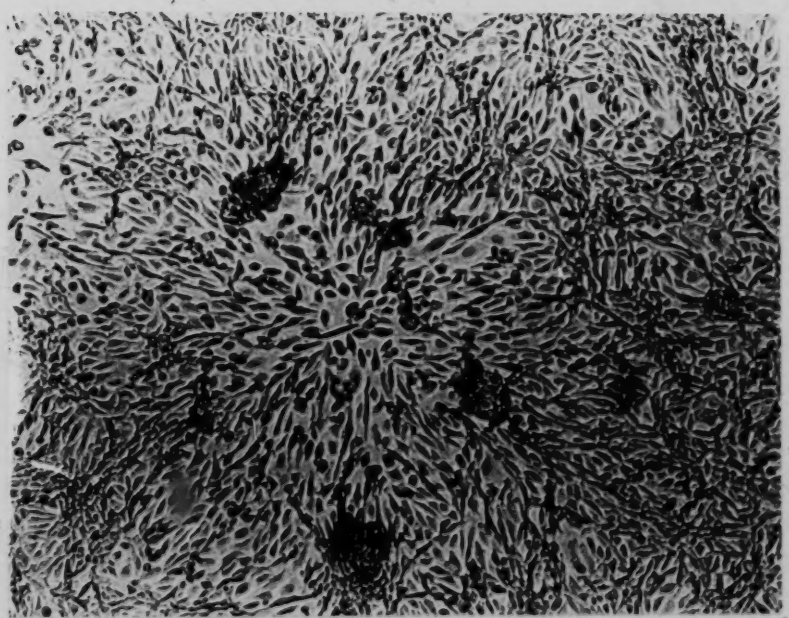


Figure 27.

Immunofluorescent staining of Halle UP-2 cells with P monoclonal antibody (16AF10). P protein staining of a 39°C culture illustrating the fine homogeneous cytoplasmic staining and the few dots/globules.

Figure 28.

Immunofluorescent staining of Halle UP-2 cells with NP monoclonal antibody (16AC5)

Figure 28a.

NP staining at 39°C illustrating an extreme example of the lack of bright dot or globule inclusions.

Figure 28b.

NP staining at 32°C showing both the intensely-stained type of syncytia and the weakly-stained type of syncytia.

Figure 28c.

Intranuclear NP dots in a cell on the edge of a syncytial hole.

Figure 29.

Immunofluorescent staining of the UP-2(39) healing holes

Figure 29a.

The edge of a healing hole nonstained by the P monoclonal antibody.

Figure 29b.

The edge of a healing hole nonstained by the M monoclonal antibody.

Figure 30.

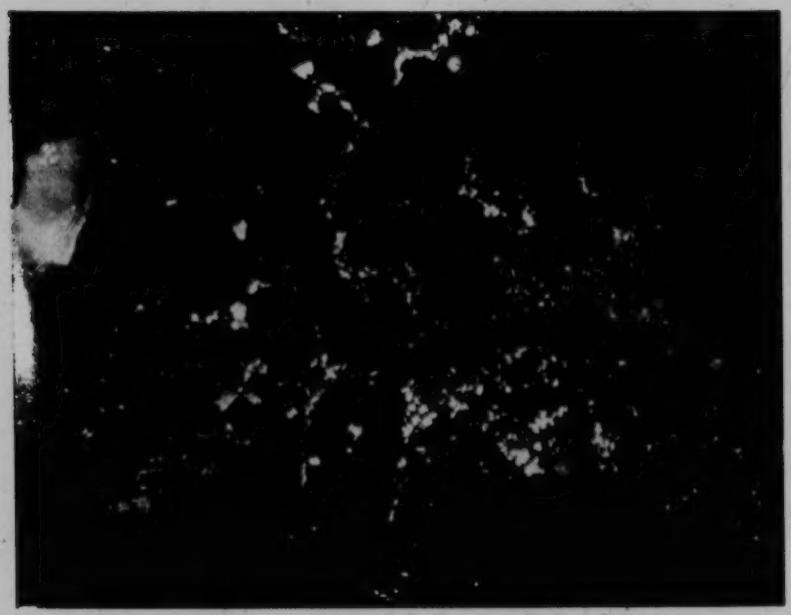
Immunofluorescent staining of Halle UP-2 passages by M monoclonal antibody

Figure 30a.

Intense cytoplasmic staining and distinct nuclear dots seen in the p147 culture at 39°C.

Figure 30b.

Bright nuclear dots seen in the p147 culture at 39°C.



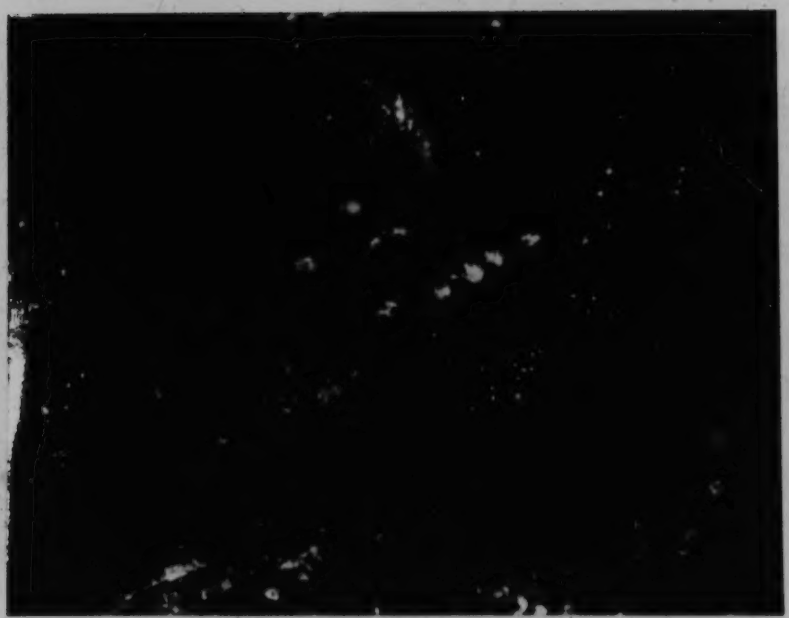


Figure 30c.

Bright cytoplasmic staining and nuclear dots of M protein seen in the p147 culture at 32°C.

Figure 30d.

A homogeneous, 8n type of syncytia seen in the p177 culture at 32°C. The diffuse nuclear staining is clearly seen.

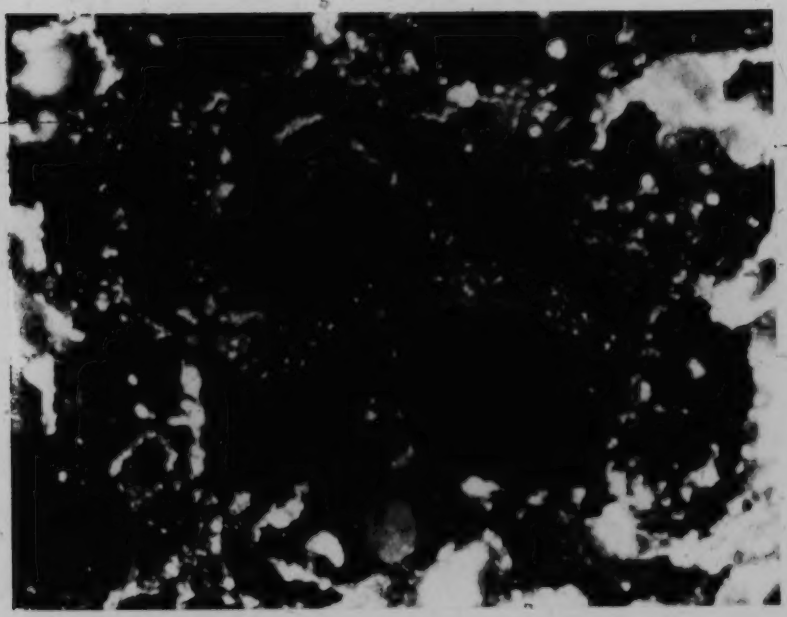


Figure 31.

Electron micrographs of Halle UP-2 cells

Figure 31a.

A beaded NB observed in the p147 UP-2(39) culture .
Magnification 55,500 X

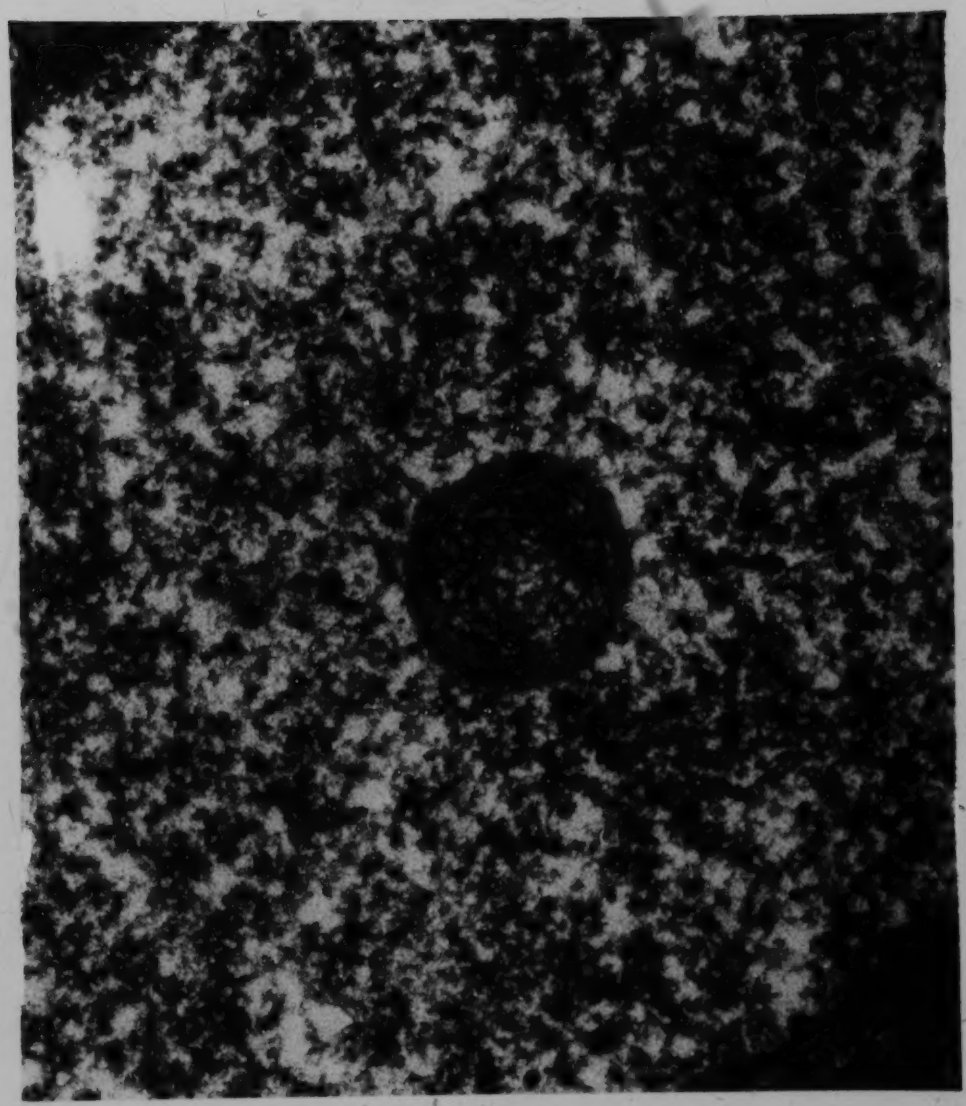
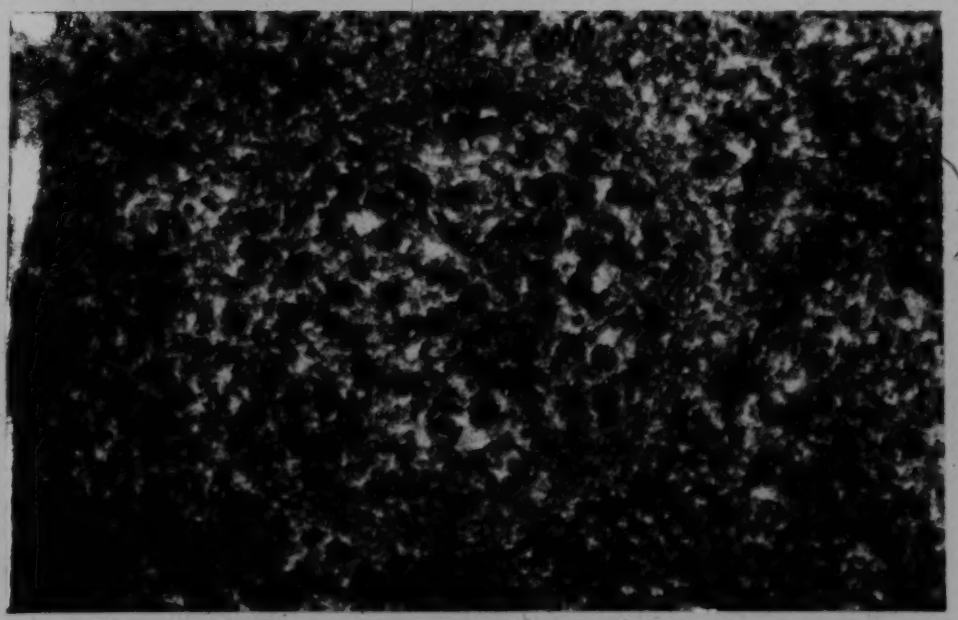


Figure 31b.

A p147 UP-2(39) cell showing extensive nucleolar destruction (nd) and a complex NB (cNB).
Magnification 32,500 X

A higher magnification of this complex NB showing the fibrillar envelope and tubular (arrow) structures inside.
Magnification 81,000 X



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7. Appendix

1. Modified Hucker's Crystal Violet

Solution A

crystal violet	2.0 grams
95% ethanol	20.0 mL

Solution B

ammonium oxalate	0.8 grams
distilled water	80.0 mL

Mix A and B, store for 24 h and filter.

2. LX-112 Embedding Resin

Mixture A

LX-112 resin[1]	36.4 grams
NMA[2]	33.6 grams

Mixture B

LX-112 resin	17.3 grams
DDSA[3]	22.7 grams

Mixture A and B are each mixed for 30 min with a magnetic stirrer. Mixture A is added to mixture B and while stirring, 3 mL of DMP-30[4] is gradually added at the level of the stirring bar using a disposable pipette. This mixture is stirred for 30 min, avoiding the incorporation of air bubbles. The resin is stored at -70°C in disposable 10 mL syringes.

[1] Ladd Research Industries Inc., Burlington, Vermont.
[2] Nadic methyl anhydride, Ladd Research Industries.
[3] Dodecenyl succinic anhydride, J. B. EM Services Inc., Pointe-Claire-Dorval, Quebec.
[4] 2, 4, 6 - tri (dimethylaminomethyl) phenol, Fisher Scientific Co..

3. Lead Citrate

lead citrate	0.01 - 0.04 grams
water	10 mL
10 M sodium hydroxide	0.1 mL

Add lead citrate to 10 mL of water in a screw-capped tube, add dropwise the sodium hydroxide and shake the tube vigorously until all the lead citrate is dissolved. Store in 1 mL syringes.

4. 0.1 M Phosphate Buffer, pH 7.2 - 7.4

Solution A

sodium phosphate, dibasic	7.1 grams
water	0.5 L

Solution B

potassium phosphate, monobasic	6.8 grams
water	0.5 L

Seven parts of A mixed with 3 parts of B gives a pH of 7.2 - 7.4.

5. PBS

sodium chloride	8.00 grams
potassium chloride	0.2 grams
monobasic potassium phosphate	0.2 grams
dibasic sodium phosphate	1.14 grams

Dissolve the above in water to a final volume of 1 L.